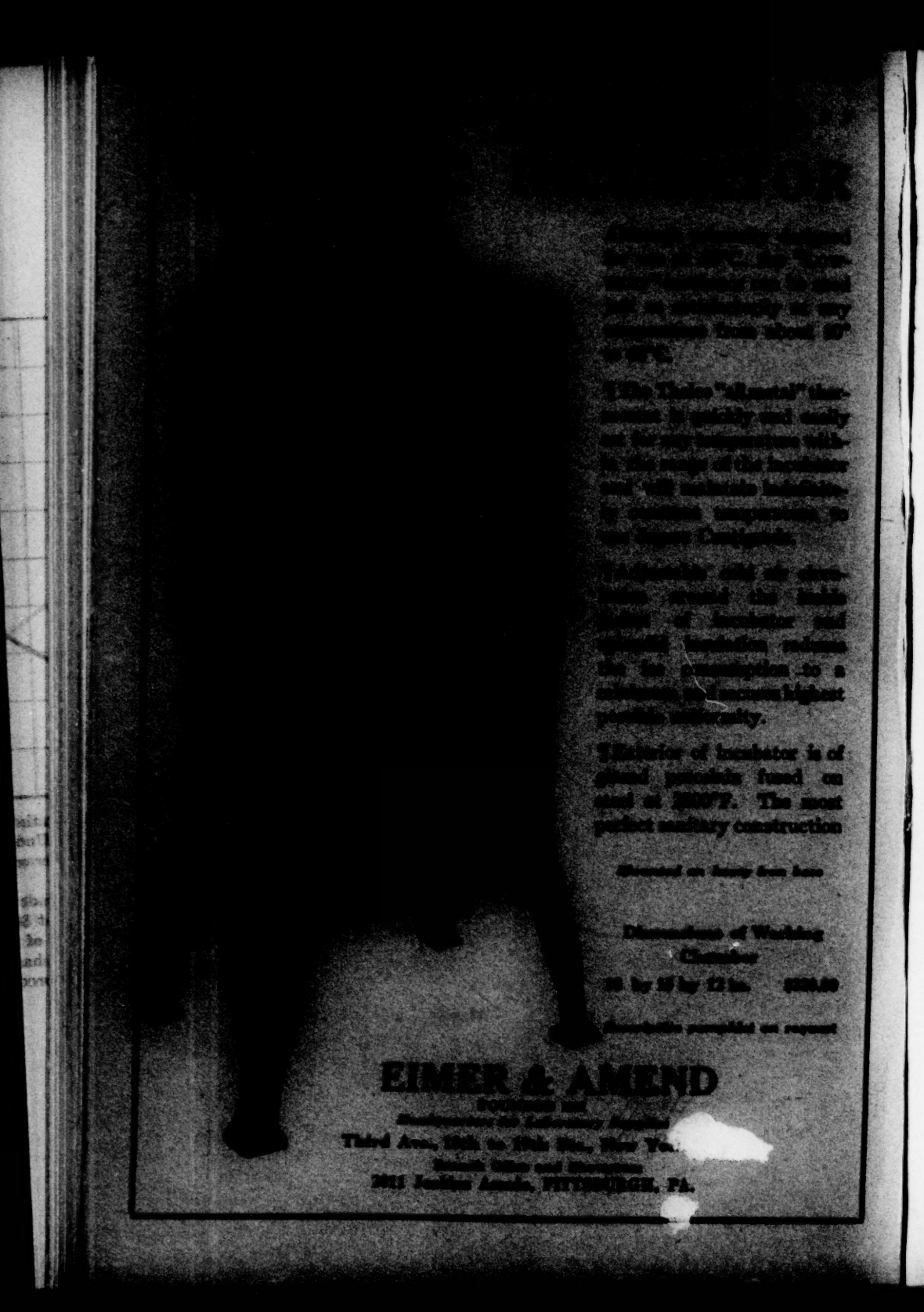
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A FOAM INHIBITOR IN THE VAN SLYKE AMINO NITROGEN METHOD.

By H. H. MITCHELL and H. C. ECKSTEIN.

(From the Department of Animal Husbandry, University of Illinois, Urbana.)

(Received for publication, January 11, 1918.)

This laboratory has experienced considerable difficulty recently in finding a suitable substance to prevent foaming during the determination of aliphatic amino nitrogen in animal and plant extracts according to the Van Slyke nitrous acid method. Kahlbaum's secondary caprylic alcohol, recommended by Van Slyke, cannot be obtained on the market now, while other preparations we have tried have invariably affected the volume of gas not absorbable by the alkaline permanganate solution. Thus, 0.2 cc.[1] of the secondary caprylic alcohol obtained from castor oil by refluxing with NaOH and subsequently distilling and fractionating,[2] gave 1.00 cc. more of gas not absorbable by alkaline permanganate in an amino nitrogen determination, than was obtained in a duplicate determination in which no alcohol was used. A preparation of caprylic alcohol obtained from Eimer and Amend gave similar results, even when fractionally distilled. We attempted to overcome this effect by shaking the alcohol with glacial acetic acid and a 30 per cent solution of $NaNO_2$ previous to using, and were able in this way to reduce the effect to 0.3 cc. of residual gas. If the alcohol, treated in this manner, was run in with the reagents and shaken with them during the removal of the air from the apparatus, instead of being run in with the sample as is customary, the effect could be reduced still further. Unfortunately, however, this preliminary treatment of the alcohol seemed to deprive it largely if not entirely of its foam-inhibiting character and therefore cannot be recommended. These experiments are interesting in showing that the vitiating effect of these caprylic alcohol preparations on the determination occurs

[1] This amount of foam inhibitor is of course excessive in routine work.

[2] Lee, O. I., *St. Luke's Hosp. Med. and Surg. Rep.*, 1917, iv; abstracted in *Chem. Abst.*, 1917, xi, 2808, 3027.

as the result of interactions with the reagents in the decomposition chamber of the apparatus.

We have been able to confirm the conclusion of Van Slyke that amyl alcohol also increases the gas volumes in amino nitrogen determinations, though its effect on the blank is negligible. The excess gas due to the amyl alcohol seems to be roughly proportional to the amount of amino nitrogen in the sample.

Lee's work[2] on foam inhibitors for the urease method of determining urea, in which the solutions are subjected to rapid aeration for the removal of the ammonia formed, lead him to recommend a mixture of 70 per cent phenyl ether and 30 per cent amyl alcohol. He claims that this mixture is more effective than either substance alone or than caprylic alcohol. We have tried this mixture of Lee's in the Van Slyke method, only to find that an effect was produced on the gas volumes obtained in the course of actual determinations, though no effect on the blank could be detected. Phenyl ether alone, however, proved to be without effect, as the following experiments indicate.

10 cc. of an asparagine solution produced in two determinations 16.70 and 16.80 cc. of gas. With 0.2 cc. of phenyl ether and 10 cc. of this solution, two other determinations under the same conditions of temperature and pressure gave 16.69 and 16.72 cc. of gas. Two other determinations in which the phenyl ether was replaced by Lee's foam-inhibiting mixture gave 17.18 and 17.19 cc. of gas. Evidently this effect is produced by the amyl alcohol.

In another experiment a solution of Kahlbaum's leucine was used. 10 cc. portions of this solution gave 9.70 and 9.73 cc. of gas in two determinations in which no foam inhibitor was used. With 0.2 cc. of phenyl ether, two more determinations under the same conditions gave 9.75 and 9.73 cc. of gas.

Phenyl ether thus appears to have no effect on the volume of gas produced in a Van Slyke determination, even when present in excess. Furthermore, according to our experience it is a very effective foam inhibitor, more effective than either amyl or secondary caprylic alcohol. Certain solutions that have foamed excessively with amyl alcohol added in amounts as large as 0.5 cc., during a Van Slyke determination, can now be analyzed with the addition of only two drops of phenyl ether, with very little if any foaming, even when the apparatus is shaken over 300 times per minute.

Besides being an effective foam inhibitor with no detrimental effects, phenyl ether may be recommended for this purpose because it can be readily synthesized at comparatively low cost. We have used the method of Ullmann and Sponagel[3] with satisfactory results.

A convenient procedure is as follows: Into a 1.5 liter round-bottomed flask are weighed 560 gm. of bromobenzene, 420 gm. of phenol, 221 gm. of KOH, and 3.5 gm. of copper-bronze. This mixture is heated on an oil bath at 210–230°C. for about 2.5 hours under a reflux condenser. We have used as a condenser a 30 inch glass tube of $\frac{3}{4}$ inch bore, topped by a water condenser of equal length. Even with this arrangement it is difficult if not impossible to prevent some loss of bromobenzene, especially during the early stages of heating. The mixture is then distilled with steam. The distillate is separated in a separatory funnel and the heavy oil at the bottom fractionally distilled. The boiling point of phenyl ether is 252.3°C. We have taken off fractions from 244–261°C. for use.

The yield may be increased greatly, at least if the steam distillation has not been carried to completion,[4] by extracting the residue from the steam distillation, in small portions, with ether, three washings for each portion being sufficient. The ether extracts are then distilled. A careful and repeated fractionation of the oil from the steam distillation and of the material extracted by ether from the residue is advantageous. After several fractionations it will be found that most of the material falls into two fractions; namely, from 150–168°, and from 244–261°C., the former fraction being bromobenzene and the latter phenyl ether. From the quantities of chemicals given above, we have obtained 287 gm. of phenyl ether, representing a yield of 47.5 per cent of the theoretical, figured on the basis of 560 gm. of bromobenzene, or of 73 per cent when from the amount of bromobenzene taken that recovered in the final fractional distillation (200 gm., boiling from 150–168°) is deducted. The relatively large amount of bromobenzene thus recovered would suggest that the time of refluxing at 210–230° could be lengthened considerably to advantage.

Phenyl ether melts at 28°C., but when in the liquid state it may be supercooled considerably without solidifying. We have observed no solidification of our product at temperatures above 20°C.

[3] Ullmann, F., and Sponagel, P., *Ber. chem. Ges.*, 1905, xxxviii, 2211.

[4] Complete steam distillation of the above mixture would consume too much time. We continued the process for about 4 hours, at which time the condensed water was still somewhat cloudy. Our experience suggests that the steam distillation may well be omitted and the product recovered from the mixture after refluxing, by ether extraction entirely.

STUDIES OF THE BLOOD FAT AND LIPOIDS OF THE DOG BEFORE AND AFTER THE PRODUCTION OF EXPERIMENTAL ANEMIA.

By HARRY DUBIN.

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(Received for publication, January 5, 1918.)

By the use of the nephelometric and colorimetric methods of Bloor,[1] blood fats have been estimated in one dog and the results show that in trypanosome anemia, the total fats are increased, while the lecithin and cholesterol are decreased. These results are in accord with the findings of Bloor[2] in pernicious anemia associated with carcinoma of the stomach.

Fats and Lipoids of Dog's Blood before and after Infection with Trypanosoma equiperdum.

Period.	Date.	Total fat.		Lecithin.		Cholesterol.		Remarks.
		Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	
	1917	*gm.**	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Normal.	Apr. 26	0.55	0.56	0.43	0.40	0.25	0.30	Apr. 24, hemoglobin, 99.
	" 28	0.52	0.53	0.40	0.41	0.29	0.26	
	" 30	0.50	0.52	0.45	0.32	0.25	0.27	
	May 2	0.53	0.57	0.37	0.35	0.22	0.27	Infected May 4.
Anemic.	May 16	0.68	0.69	0.38	0.30	0.22	0.19	May 18, hemoglobin, 56.
	" 18	0.70	0.75	0.29	0.27	0.20	0.23	
	" 22	0.71	0.78	0.31	0.25	0.14	0.12	

* All figures represent gm. per 100 cc. of blood.

[1] Bloor, W. R., Studies on blood fat. II. Fat absorption and the blood lipoids, *J. Biol. Chem.*, 1915, xxiii, 317; The determination of cholesterol in blood, 1916, xxiv, 227; Fat assimilation, xxiv, 447.

[2] Bloor, The distribution of the lipoids ("fat") in human blood. *J. Biol. Chem.*, 1916, xxv, 577.

ANIMAL CALORIMETRY.

THE INFLUENCE OF MECHANICAL WORK UPON PROTEIN METABOLISM DURING THE HEIGHT OF MEAT DIGESTION IN THE DOG.

FOURTEENTH PAPER.

BY H. V. ATKINSON.

(From the Physiological Laboratory, Cornell University Medical College, New York City.)

(Received for publication, January 16, 1918.)

In the last paper of this series[1] the heat production of a dog was measured, while he was running, during the 4th and 5th hours after the ingestion of 750 gm. of meat. As the dog would not retain urine during a period of exercise, it was necessary to assume, in making the calculations of the metabolism of the period, that the protein metabolism was the same as that which obtained in the same dog at rest after giving the same quantity of meat. However, this assumption might have been false because, on the one hand, the largely increased general blood flow might have furnished the gut with a notably increased volume of blood, thereby accelerating the absorption of amino-acids, or, on the other hand, one might conceive that the demands of the working muscles for blood might so decrease the supply of blood available for the intestines that a marked retardation in the absorption process might have ensued.

A fox-terrier female weighing about 10.5 kilos was found to be willing to eat at most 600 gm. of meat daily at one meal, and upon this quantity the animal was maintained throughout the experiment. As it was found that the animal would not hold urine while running on the treadmill for a period of 1 hour, the period of active exercise was reduced to half an hour, though the urine was collected for the whole hour.

A preliminary series of experiments showed that the maximal quantity of urinary nitrogen was eliminated during the 5th hour after administering meat, as appears in the following table.

[1] Anderson, R. J., and Lusk, G., Animal calorimetry, XIII, *J. Biol. Chem.*, 1917, xxxii, 421.

TABLE I.

The Hourly Elimination of Nitrogen during the 4th, 5th, and 6th Hours after Administering 600 Gm. of Meat to a Resting Dog.

Date.	N in urine per hr.		
	4th hr.	5th hr.	6th hr.
	gm.	*gm.*	*gm.*
Oct. 4, 1917	1.00	1.08	1.05
" 5 "	1.01	1.04	1.06
" 13 "	1.06	1.11	1.11
" 14 "	1.01	1.13	1.02
Dec. 29 "	1.07	1.05	1.08
Jan. 2, 1918	1.05	1.07	1.09
Average	1.04	1.08	1.07

The dog was then caused to run on the treadmill for 30 minutes in four experiments during the beginning of the 5th hour, and then in two later experiments during the end of the 5th hour. The following results were obtained.

TABLE II.

The Influence of a Run of Half an Hour during the 5th Hour after the Administration of 600 Gm. of Meat upon the Nitrogen Elimination of That Hour Contrasted with That of the Previous and Subsequent Hours When No Work Was Done.

Date.	N in urine per hr.		
	4th hr.	5th hr.	6th hr.
	gm.	*gm.*	*gm.*
Oct. 25, 1917	1.07	1.09*	—
Nov. 3 "	1.02	1.08†	1.06
" 6 "	1.09	1.12†	1.11
" 8 "	1.05	1.05†	1.04
Dec. 31 "	1.06	1.07†	1.08
Jan. 3, 1918	1.04	1.03†	1.10
Average	1.05	1.07	1.07

* Distance traveled, 2,090 meters = 1.30 miles.
† Distance traveled, 2,125 meters = 1.32 miles.

It is clear, on comparison of Tables I and II, that *mechanical work has no influence on the hourly rate of absorption of protein or on the intensity of the hourly metabolism of protein in a dog which has been given meat in large quantity.*

NOTES ON THE DIRECT DETERMINATION OF UREA AND AMMONIA IN PLACENTA TISSUE.

By FREDERICK S. HAMMETT.

(*From the Department of Anatomy of Harvard Medical School, Boston.*)

(Received for publication, January 14, 1918.)

INTRODUCTION.

Certain questions as to method of analysis have come up during the course of some work on a study of the urea content of placenta tissue. From the point of view of routine work the method of Sumner (1) comes nearest to the analytical ideal of combined accuracy, speed, and minimum of manipulation. Inasmuch as the results herein reported have been obtained by following his procedure no recapitulation is necessary, any alterations being considered in detail in the text.

Urease.

The application of the urea-splitting enzyme of the soy bean, discovered by Tacheuchi (2) and intensively studied by Van Slyke and Cullen (3, 4), to the determination of urea in blood and tissues was first made by Marshall (5). I have used, however, an extract, prepared in Folin's laboratory, of the jack bean whose urease content was recorded by Mateer and Marshall (6, 7). This extract has proved satisfactory in every way.

Potassium Carbonate.

Fiske (8) and Van Slyke and Cullen (3) advocate the use of highly concentrated solutions of potassium carbonate for the liberation of ammonia. It is more advantageous, however, with placenta tissue to use the solid salt, inasmuch as the volume of the contents of the test-tube is already large due to the water used in rinsing down and suspending the pulped tissue. Considerable variations in the quantity employed are recorded in the literature ranging from 5 (1) to 12 (9) gm. of solid carbonate. Van Slyke and Cullen (3) determined that the use of less than 1 gm. of carbonate for each 2 cc. of solution necessitates a longer aeration.

I have found that 5 gm. of solid carbonate is amply sufficient

to cause complete liberation of the ammonia from as much as 20 cc. of solution when aerated vigorously for 30 minutes. In view of the increasing difficulty in obtaining potassium salts as well as their increasing cost and the necessity for conservation, minimal amounts consistent with adequate action are indicated. Table I shows the results of a series of comparative tests when using 5 and 10 gm. of solid carbonate, other conditions being equal.

In view of the fact that large variations in the amounts of NH_3 in the tissue suspensions might cause appreciable variations in the efficiency of the amounts of carbonate used, advantage was

TABLE I.

Relative Quantity of NH_3 Liberated from Placenta Tissue Suspensions in 15 Cc. of Solution When Using 5 and 10 Gm. of K_2CO_3 and Strong Aeration for 30 Minutes.

NH_3 in 100 gm. of placenta tissue.	
5 gm. K_2CO_3.	10 gm. K_2CO_3.
mg.	*mg.*
22.8	23.0
16.3	15.8
13.9	14.4
12.3	11.9
11.4	11.6
7.6	7.6
4.9	4.8
4.8	4.6
4.7	4.7

taken of the NH_3-forming ability of urease to produce in some of the suspensions higher NH_3 values. Accordingly, some of the figures represent not only the ammonia as such of the tissue, but also the ammonia liberated from the urea.

It is quite plain that 5 gm. of potassium carbonate is a sufficient quantity for general use.

Time and Vigor in Aeration.

If two samples of the same tissue differing in weight are aerated slowly for 2 minutes and then vigorously at the rate of 5 liters a minute for the remainder of 30 minutes, the amount of ammonia blown over is proportional to the amount of tissue taken for

analysis. This lends additional support to the contention of Van Slyke and Cullen (3) that longer periods of aeration as advanced by Fiske (8) and others are unnecessary. Table II gives a few of the results demonstrating this point.

Repeated tests have been unable to demonstrate the carrying over into the receiving flask of any alkaline spray from the aerated mixture mentioned by Sumner (1) as a reason for diminished vigor in aeration. A comparison of the traps used by Sumner and those of this laboratory shows a markedly higher effi-

TABLE II.

NH_3 Recovered from Varying Amounts of Placenta Tissue When Uniformly Aerated for 30 Minutes.

Specimen No.	Weight of tissue.	100 Gm. of tissue.		
		0.01 N NH_3	0.01 N HCl	NH_3
	gm.	*cc.*	*cc.*	*mg.*
10	9.95	8.60	10	2.4
	7.17	8.97	10	2.4
46	6.65	8.40	10	4.1
	5.12	8.76	10	4.1
29	7.29	6.00	10	9.3
	5.97	6.71	10	9.4
46 a	7.13	5.80	20	33.8
	4.45	11.12	20	33.9

ciency in this respect for the latter. Foaming is easily prevented in the aerated mixture by the addition of 1 cc. of a solution containing equal parts of amyl alcohol, toluene, and ethyl alcohol. The use of long-necked 100 cc. flasks prevents loss by spattering, and the foaming in these containers is reduced to a minimum by the addition of a few drops of the above mixture from time to time.

Factors Influencing the Ammonia Fraction in the Urea Determination.

The quantity of urea in any given sample is usually determined by the difference between the ammonia already present and the total ammonia blown over after the action of urease. Having

standardized conditions of aeration, enzyme action, and quantity of carbonate used, the changes in the ammonia content of the tissue during standing while undergoing fermentation introduce a considerable error.

Folin and Denis (10) early pointed out that the ammonia content of drawn blood increased if the sample was allowed to stand. Although Mateer and Marshall (6) have definitely directed that in the determination of urea by the urease method ammonia determinations need to be made simultaneously and similarly, only excluding the urease preparation, various published reports have failed to mention or consider ammonia determinations; Slemons and Morriss (11) have even gone so far as to state that such analyses yield insignificant results.

TABLE III.

Increase in the Quantity of NH_3 Recoverable from Placenta Tissue after Standing Half an Hour.

NH_3 in 100 gm. of tissue.	
Immediate.	After ½ hr.
mg.	*mg.*
6.5	7.4
5.9	6.8
2.3	2.9
3.0	4.1
1.7	2.8
1.2	1.9

In order to determine whether or not there was any marked difference between the ammonia obtained by immediate aeration and that given off from the placenta tissue after it had stood for ½ hour in suspension in the test-tube, a series of tests was made, the results of which are found in Table III.

It is evident that increases in the ammonia content of the tissues ranging from 14 to 85 per cent may occur, which alone indicates the necessity of allowing the tissue from which the ammonia values are to be determined, as a basis of the analysis for urea, to stand under similar conditions as do those samples in which the urea is undergoing splitting by the urease.

The results reported in this work are based on parallel determinations and have an average variation of 2 per cent.

SUMMARY.

1. In the determination of ammonia or urea in placenta tissue the use of solid potassium carbonate offers opportunity to maintain a minimum volume of the aerated mixture.

2. 5 gm. of the solid potassium carbonate are sufficiently effective in liberating the ammonia present in amounts of tissue ranging from 4 to 10 gm. and suspended in 10 to 15 cc. of water.

3. 2 minutes of mild aeration followed by 28 of vigorous passage of air completely drives over all the ammonia present in the amounts of tissue ordinarily used for analysis.

4. The use of a mixture of equal parts of amyl alcohol, toluene, and ethyl alcohol prevents foaming in test-tube and flask.

5. The ammonia content of tissue increases during standing for ½ hour.

6. In the direct determination of urea in tissues by means of the urease method it is imperative that simultaneous analyses be made for the ammonia fraction. The tissue samples from which the ammonia values are to be obtained must be allowed to digest for the same length of time and under the same conditions as do those specimens from which the urea is determined.

Through the courtesy of Professor Otto Folin the work reported in this paper was carried on in the Biochemical Laboratories of the Harvard Medical School.

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5. Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.
6. Mateer, J. G., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1916, xxiv, p. xxx.
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11. Slemons, J. M., and Morriss, W. H., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 343.

COMPARISON OF THE GLUCOSE AND CHOLESTEROL CONTENT OF THE BLOOD.

By F. H. McCRUDDEN and C. S. SARGENT.

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(Received for publication, January 4, 1918.)

The association of hyperglycemia and hypercholesterolemia in diabetes[1,2] and of hypoglycemia and hypocholesterolemia in progressive muscular dystrophy,[3,4] pointing to some kind of physiological relationship between glucose and cholesterol, suggested that there might be a parallelism between the amounts of the two substances in the blood. But this appears not to be the case (Table I and Chart 1).

Glucose was determined by the method of Lewis and Benedict,[5] and cholesterol by the method of Autenrieth and Funk.[6] Analyses were made in duplicate or triplicate.

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[1] Bloor, W., The lipoids ("fat") of the blood in diabetes, *J. Biol. Chem.*, 1916, xxvi, 417.

[2] Joslin, E. P., Bloor, W. R., and Gray, H., The blood lipoids in diabetes, *J. Am. Med. Assn.*, 1917, lxix, 375.

[3] McCrudden, F. H., and Sargent, C. S., Hypoglycemia and progressive muscular dystrophy, *Arch. Int. Med.*, 1916, xvii, 465.

[4] McCrudden and Sargent, Chemical changes in the blood and urine in progressive muscular dystrophy, progressive muscular atrophy, and myasthenia gravis, *Arch. Int. Med.*, 1918, xxi, 252.

[5] Lewis and Benedict's method (*J. Biol. Chem.*, 1915, xx, 61) as modified by Myers and Fine (Myers, V. C., and Fine, M. S., Chemical Composition of the Blood in Health and Disease, New York, 1915.)

[6] Authenrieth, W., and Funk, A., Ueber kolorimetrische Bestimmungsmethoden: die Bestimmung des Gesamtcholesterins im Blute und in Organen, *Münch. med. Woch.*, 1913, lx, 1243.

TABLE I.

Case.	Condition.	Glucose.	Cholesterol.
		mg. per 1 gm.	*mg. per 1 gm.*
1	Chronic arthritis	0.086	1.32
2	" "	0.0862	1.50
3	Paralysis agitans	0.088	1.45
4	Chronic arthritis	0.088	2.01
5	" "	0.088	2.09
6	" "	0.0896	1.45
7	Psoriasis	0.090	1.53
8	Pernicious vomiting	0.092	2.22
9	Chronic arthritis	0.098	1.41
10	Bone tuberculosis	0.100	2.07
11	Chronic arthritis	0.101	1.43
12	" "	0.103	1.94
13	" "	0.104	1.55
14	" "	0.106	1.69
15	" "	0.106	1.88
16	Normal	0.109	2.09
17	Chronic arthritis	0.111	2.11
18	Normal	0.115	1.70
19	Chronic arthritis	0.117	2.26
20	Multiple sclerosis	0.120	1.53
21	Arteriosclerosis	0.120	1.54
22	"	0.123	1.59
23	Chronic nephritis	0.123	2.30
24	" arthritis	0.124	1.57
25	" "	0.125	1.55
26	" endocarditis	0.127	1.69
27	" arthritis	0.128	1.29
28	Normal	0.129	1.71
29	Chronic arthritis	0.131	1.45
30	" nephritis	0.133	1.17
31	Paralysis agitans	0.137	2.01
32	Chronic arthritis	0.145	2.35
33	" nephritis	0.147	1.96
34	Lues	0.140	1.71
35	Progressive muscular atrophy	0.179	1.70
36	Chronic nephritis	0.204	1.75

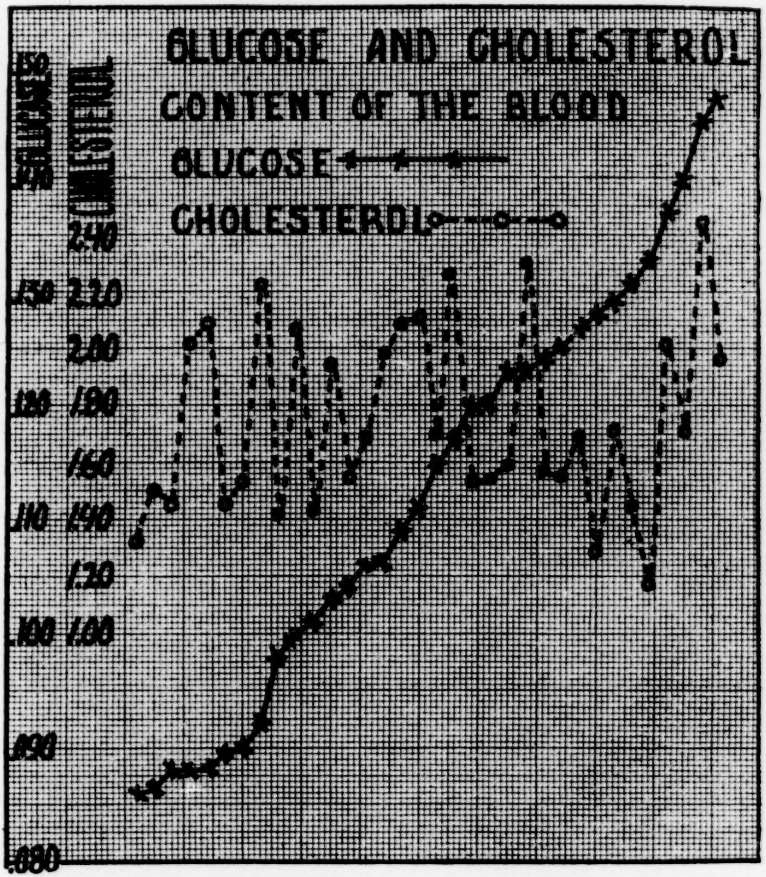

CHART 1. The crosses connected by a full line show the glucose content in per cent of the blood of thirty-four patients (arranged in order of magnitude).

The circles connected by a broken line show the cholesterol content of the blood, also in per cent, in the same cases.

THE ANALYSIS OF MILK SECRETED BY A SUCKLING DOE KID.

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(Received for publication, January 18, 1918.)

The literature written on the secretion of colostrum contains various theories advanced to account for the appearance of colostrum instead of normal milk immediately following parturition. Some authorities believe that imperfect functional activity of the mammary glands at this time is responsible for the secretion of a fluid so different in composition and consistency from normal milk. Still others are of the opinion that the newly born require a food of the nature of colostrum for the first few feedings before they receive milk; that the colostrum was provided by nature to compensate for this need.

In the newly born there is sometimes a secretion from the mammary glands known as "witches milk." This fluid has a qualitative resemblance to milk but differs widely quantitatively from normal milk. It is often secreted at birth but disappears soon afterward. There have been instances in which young animals have been reported as secreting milk usually as a result of mechanical manipulation of the udder or by being suckled. Whether the first milk so secreted was normal milk, colostrum, or of the nature of "witches milk" is not, to my knowledge, recorded.

On May 2, 1917, a one-half breed Saanen milk goat at this Station was delivered of twin doe kids. One kid was sold when about 3 months old. The other kid appeared perfectly normal but was observed to have an exceptionally well developed udder when about only 2 months old. Its udder was not manipulated, suckled, or treated in any way to stimulate its development. When 4 months and 4 days old, on September 6, 1917, its udder

had the appearance of containing milk. Upon examination it was found to contain milk, which was withdrawn and analyzed. A yield of 120 cc. was obtained and proved to be perfectly normal milk, as is shown in the table. Since this secretion was of a spontaneous nature and had not been initiated by any applied stimulus it was decided to remove the milk at intervals several days apart in order to avoid any mechanical stimulation due to repeated milkings. From the following table the normal character of all the milk secreted can be clearly seen.

Chemical Analyses of Milk from a Virgin Kid.

Date.	Fat.	Protein.	Ash.	Total solids.	Sugar.	Milk.	Remarks.
1917	per cent	per cent	per cent	per cent	per cent	cc.	
Sept. 6.....	4.20	3.60	0.84	13.32	4.68	110	Kid, 4 mos., 4 days old.
" 15.....	4.40	3.58	0.82	13.44	4.61	90	
" 22, 23, 24.	4.32	3.53	0.84	13.22	4.53	—	Composite.
" 29.....	4.78	3.36	0.81	13.52	4.57	90	
Oct. 3.....	4.52	3.32	0.75	13.56	4.97	110	
" 5.....	5.60	3.43	0.80	14.20	4.37	75	
" 13.....	5.41	3.51	—	—	—	130	
" 29.....	4.82	—	—	—	—	185	
Nov. 11.....	—	—	—	—	—	240	
" 21.....	—	—	—	—	—	295	
" 29.....	—	—	—	—	—	175	
Dec. 2.....	—	—	—	—	—	100	
" 11.....	4.17	3.94	0.78	13.09	4.20	175	
" 17.....	4.70	4.08	0.80	13.87	4.29	125	

The physical character of the milk is practically the same as normal goat's milk. The curd obtained by the use of rennin or pepsin is, however, much finer and softer than that obtained from any other of the goat's milk examined. The milk was substituted in the diet of an 11 months' old baby for normal goat's milk without any detectable difference either in the appetite or physical condition of the baby. The milk cannot be distinguished by odor or flavor from any other sample of goat's milk. It has a very low bacterial content and keeps for an exceptionally long period. The relative percentage of casein and albumin is practically identical with normal goat's milk. The work is being continued, and any

changes in the composition or character of the milk will be noted as they appear.

SUMMARY AND CONCLUSIONS.

A 4 months' old virgin doe kid spontaneously commenced to secrete milk having all the characteristics, properties, and chemical composition of the milk secreted by its mother and other goats under observation. At no time, from the earliest secretion of milk to the present time, has there been any colostrum secreted. This would indicate that the secretion of colostrum is associated with and possibly is produced by the cessation of pregnancy and may not occur in lactation not associated with pregnancy.

THE CATALASE CONTENT OF ASCARIS SUUM,* WITH A SUGGESTION AS TO ITS RÔLE IN PROTECTING PARASITES AGAINST THE DIGESTIVE ENZYMES OF THEIR HOSTS.

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(Received for publication, January 8, 1918.)

Many explanations have been offered as to why gastric and intestinal parasites are not digested by their hosts. A brief survey and discussion of many theories can be found in text-books on physiology, for this question resolves itself into the time-worn query, Why is living tissue not digested? The theory which has perhaps gained most attention is that anliferments or antienzymes protect living matter, and this theory has been used by Weinland (1903) to explain the resistance of intestinal parasites to the destructive influence of digestive enzymes. Upon rubbing up *Ascaris* into a pulp and extracting with alcohol he obtained a substance which he termed *Antifermente* because he claimed that this substance protected the proteins from the proteolytic action of pepsin and trypsin. On the basis of his own interpretation this theory was open to criticism because he had to kill the worms to obtain the substance, and it was argued that if the antiferment existed after death it should protect the dead worms as well as the living. More recent work on the antienzymes has offered very good explanations for these phenomena, but it is not the intention to discuss these intricate facts.

In recent times Burge and Burge (1915, *a*) have advanced another theory which might be termed an "oxidation theory," and they have used this not only to explain the resistance of intestinal parasites to the digestive action of their hosts, but maintain that living cells protect themselves from the destructive enzymes by means of the oxidation processes going on in them. It has been shown that the mucosa of the stomach and intestines possesses intense oxidative properties, and Burge (1914) has shown that the digestive enzymes are very easy to oxidize and are then inactive. By applying these facts to intestinal parasites he was able to demonstrate that living worms are not digested while dead worms are, and if the dead ones are permeated with nascent oxygen they withstand

* It is not unlikely that *Ascaris suum* and *Ascaris lumbricoides* are identical.

the attack of the digestive enzymes. It should be noted that he killed the worms by electrolysis, a method by which enzymes may be destroyed (Burge and Burge, 1915, *b*).

In addition to these interesting facts another group of phenomena has accumulated. It has been shown (Burge, 1916) that the catalase content of muscles is highest in those in which more work is done, *i.e.* in which more oxidation is taking place; that during starvation the catalase content of the fat and muscles, with the exception of the heart, is less than normal (Burge and Neill, 1917); that the catalase content of the liver, heart, and blood is decreased in phosphorus poisoning and that the tissues are autolyzed in the same proportions that they lose catalase (Burge, 1917).

It occurred to the author that if this theory was valid and the presence of oxidative processes in the intestinal parasites protected them from digestion, then the body walls of these parasites should yield comparatively large amounts of catalase, if the amount of catalase in the tissue was an indication of the oxidative processes. The common *Ascaris*, a roundworm, from the hog was used in a series of experiments to determine the amount and distribution of catalase in the body.

The method used was one adapted from ordinary gas analysis. It seems to have advantages over the one used by Burge (1916) in that the gas may be collected under standard conditions, the apparatus is easier to handle, is neater, and perhaps more accurate, although it must be borne in mind that the determination is not an absolute one but comparative. The text-figure shows the apparatus. The material was washed with a 0.75 per cent sodium chloride solution and finely chopped up, weighed in a crucible, and introduced into the bottle C in which had been put 25 cc. of one-half diluted commercial hydrogen peroxide. The pinch-cock E was opened and the bottle stoppered as indicated in the figure, connecting the bottle to the burette, which was filled with water. The stop-cock B was then opened and the water in the burette D leveled to the zero mark by raising or lowering the bulb A. When this was done the pinch-cock was closed, the crucible upset in the hydrogen peroxide, and the bottle C was shaken for 10 minutes. At the end of that time the stop-cock B was closed and the water in the leveling bulb brought to the level of the water in the burette and the reading made. This was corrected for 760 mm. pressure and 25°C. It is obvious

that the amounts of gas determined by this method will be less than those made according to the Burge method, and Tables II and III indicate this in the case of frog tissue, run at the same time on tissue taken from the same frog, which had been washed free from blood by running 0.75 per cent sodium chloride through the blood system. It will be seen that the ratio between my

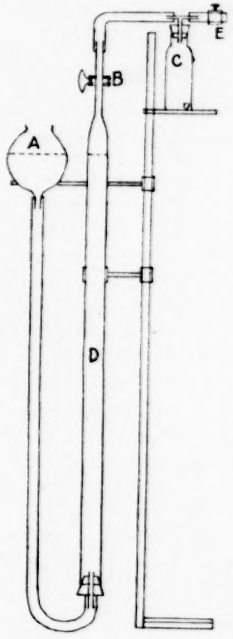

FIG. 1.

method and Burge's is 1: 1.52 in this case. A Schiff's azotometer has been used for catalase determinations (Dox, 1910).

By the method described determinations of the catalase content of the whole worm, the body wall, body fluid, and visceral organs were made. It should be recalled that the body wall of these worms consists of an outer, inert, non-cellular cuticula, a thin layer of subcuticula, and inside of this a single layer of muscle cells. The visceral organs consist in adult females, which

were the only ones used, almost entirely of the reproductive organs, in which countless numbers of embryos are rapidly being formed and hence very rapid oxidation must be taking place.

From Table I the following facts were obtained: The whole worms yield 15 cc. of oxygen per gm., the body wall 13 cc., the

TABLE I.*

Oxygen per Gm. of Ascaris suum.

Ascaris suum.	Amount used.	Sample.						Average.
		1	2	3	4	5	6	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Whole worm	1	16	14	17	15	14	16	15
Body wall	1	10	13	16				13
Body fluid	1	7	7	7				7
Visceral organs	1	23	26	30				26
Total								15

* In all the determinations in the tables 25 cc. of hydrogen peroxide were used and the material was shaken in this for 10 minutes. The number of cc. of oxygen liberated was then read.

TABLE II.

(*Author's Method.*)

Oxygen per Gm. of Rana pipiens.

Rana pipiens.	Amount used.	Sample.			Average.
		1	2	2	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*
Leg muscles	1	24	22	23	23
Ovary	1	130	120	123	124

TABLE III.

(*Burge's Method.*)

Oxygen per Gm. of Rana pipiens.

Rana pipiens.	Amount used.	Sample.			Average.
		1	2	3	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*
Leg muscles	1	43	46	42	43
Ovary	1	190	189	185	188

body fluid 7 cc., and the visceral organs 26 cc. The average of the last three items run separately yielded the same amount as the total worms, thus furnishing a check on the determinations. The cuticula, when freed from the other two layers of the body wall, does not liberate oxygen from hydrogen peroxide but weighs one-fourth the total amount of the wall; hence it will be seen that the other two layers involved really yielded 17 cc. of oxygen per gm.

While these amounts are very small as compared with those obtained from free living animals it must be remembered that these parasites are very sluggish and their activities are greatly curtailed by the limited amount of oxygen in their environment. However, when these results are compared with the case of the frog in Table II, an interesting observation can be made. A gram of muscle and subcuticula from the *Ascaris* yielded about five-eighths as much oxygen as did the reproductive organs, while in the frog the most active muscles in the body yielded only about one-fifth the amount yielded by the ovaries of that animal. In other words, the sluggish and inactive muscles of the *Ascaris* have more than three times the amount of catalase in them as the most active muscles of a very active vertebrate, when they are compared with the reproductive organs of the same animals respectively from which they came. At the present time this seems to be the most fair way to compare the two cases. It remains for someone to show quantitatively how much of this catalase is necessary to carry on the functions of nutrition, motion, etc., and finally that the amount left over is sufficient and does provide the necessary amount of oxygen to protect the worm from the enzymes of its host. The work presented in this paper, while not final, indicates, to the mind of the author, a possible connection between the two statements made by Burge, that intestinal worms are protected from enzymic action by oxidative processes, and that catalase is in some way responsible for the oxidative processes in animals other than parasites. It is hoped that this contribution will serve as a stimulus for others to undertake investigations along this line.

CONCLUSIONS.

1. Catalase may be better determined by the new method given in this paper than by the method used by Burge.

2. There is five-eighths as much catalase in the body wall of *Ascaris suum* as in the visceral organs, and one-fourth as much in the body fluid as in the visceral organs.

3. There is three times more catalase in the body wall of *Ascaris suum* than in the leg muscles of *Rana pipiens*, if one uses the amount of the catalase in the reproductive organs of each form as the units of measurement.

4. On the basis of this last statement it can be assumed that there is more than enough catalase in the body wall of this parasitic worm for its metabolic and locomotory functions, and hence it is possible that this excess is used to liberate oxygen for protecting the parasite against the digestive enzymes of its host, if Burge's theory be true.

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Burge, W. E., and Burge, E. L., The rôle of nascent oxygen in regulating the activities of enzymes in animals and plants, *Am. J. Physiol.*, 1914, xxxiv, 140.

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Dox, A. W., The catalase of molds, *J. Am. Chem. Soc.*, 1910, xxxii, 1357.

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THE FATTY ACIDS IN HUMAN BLOOD IN NORMAL AND PATHOLOGICAL CONDITIONS.

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INTRODUCTION.

As has been shown in previous researches the fatty acids in human blood exist as glycerides, cholesterol esters, soaps, or as free acids; they are also represented as a radical of lecithin and the phosphatide groups. Their content varies in continued starvation, fat absorption, anesthesia, certain pathological conditions, etc., as recently reported and confirmed in a series of papers by Bloor.[1] In normal individuals when care is taken to avoid the above conditions the amount of fatty acids in the blood is fairly constant. But little, if any attention has been given to the quality of the fatty acids, and especially to the proportion of saturated to unsaturated fatty acids as well as the degree of unsaturation.

That there is a difference in the quality of the fatty acids of the whole human body is shown by the fact that the fats called interstitial, depot, and organ fats, each absorb iodine in different proportions, thus signifying that the unsaturated part of the fatty acids varies in the different tissues. Jaeckle[2] found an iodine number of 62 to 73 for human (interstitial) fat, and 70 to 80 per cent of the fatty acids present as glycerides were oleic acid. Hartley's[3] analysis of liver fat shows the presence of higher unsaturated fatty acids than oleic acid as indicated by the iodine number of 165 to 175.

[1] Bloor, W. R., *J. Biol. Chem.*, 1913-14, xvi, 517; 1914, xix, 1. Bloor, W. R., and MacPherson, D. J., *ibid.*, 1917, xxxi, 79. Bloor, *ibid.*, 1917, xxxi, 575.

[2] Jaeckle, H., *Z. physiol. Chem.*, 1902, xxxvi, 53.

[3] Hartley, P., *J. Physiol.*, 1909, xxxviii, 353.

From the point of view of hemolytic action, we are especially interested in the unsaturated part of the fatty acids, since it has been shown by Noguchi[4] and Faust and Tallquist[5] that the higher saturated acids, palmitic and stearic, are not hemolytic either as such or as their soaps. On the other hand, the unsaturated fatty acids, as oleic acid, have a strong hemolytic action, and it has been demonstrated by Faust and Tallquist that this acid is the cause of the anemia in *Bothriocephalus latus* infection.[6] Further, Lamar[7] has shown an existing relation between the degree of unsaturation and lytic action as he was able to show that the sodium soap of linoleic acid dissolved pneumococci more rapidly in higher dilutions than sodium oleate. McPhedran,[8] working with red blood cells, was unable to demonstrate any difference in the lytic action of linoleic and oleic acids as Lamar had claimed in the case of bacteria, and concluded that there was no relationship between the lytic action and degree of unsaturation.

In my previous paper,[9] I reported that the iodine number of fatty acids of the blood in cases of pernicious anemia was not exceptionally high; but, as I stated, the matter needed further investigation, as the material used for iodine determinations was a mixture of saturated and unsaturated fatty acids; so that this finding had no other meaning than that unsaturated fatty acids were present. The mere presence of unsaturated fatty acids cannot be considered the primary cause of toxic hemolysis, since such acids exist in normal human blood, as shown below. It is therefore necessary to look further for the causative factor.

[4] Noguchi, H., *J. Exp. Med.*, 1906, viii, 87.

[5] Faust, E. S., and Tallquist, T. W., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 367.

[6] The cholesterol ester of oleic acid is hemolytic but not so strongly so as oleic acid itself. Since they demonstrated in the saponified ether extract of *Bothriocephalus latus* both oleic acid and cholesterol and were unable to show the presence of glycerol, they concluded that the oleic acid was present as a cholesterol ester without attempting to isolate it as such.

[7] Lamar, R. V., *J. Exp. Med.*, 1911, xiv, 256.

[8] McPhedran, W. F., *J. Exp. Med.*, 1913, xviii, 527.

[9] Csonka, F. A., *J. Biol. Chem.*, 1916, xxiv, 431.

Methods.

I.

The saponification of the alcohol-ether extract was preferred to the direct saponification of whole blood because of the inconvenience of working with such large quantities, and the large volumes required to saponify in alcoholic KOH medium in a concentration sufficient to be sure that the esters of cholesterol are also split.

Blood was taken from the vein before breakfast.[10] A few crystals of potassium oxalate were added to prevent clotting. 75 to 125 cc. of blood were measured with a pipette and run slowly into 250 to 400 cc. of 95 per cent alcohol with constant stirring. After the protein matter had settled it was filtered through a Buchner funnel by suction. The blood proteins were put into a large extraction shell and extracted by absolute alcohol for 24 hours and again by petroleum ether for 24 hours in a Soxhlet apparatus. The filtrate from the blood protein as well as the absolute alcohol extract was evaporated on the water bath to dryness. It was redissolved in the petroleum ether extract, filtered through cotton, measured, and about an eighth of it was used for the determination of the fat and lipoid content. From the remainder the petroleum ether was distilled off, the residue saponified, and the fatty acid determined according to the method of Gephart and Csonka.[11]

The first step in the separation of the unsaturated from the saturated fatty acids was to convert them into their lead soaps. These were extracted by ether, according to the method of Varrentrapp, which dissolves the lead soaps of the unsaturated fatty acids. This method is not strictly quantitative, but it is the best at present devised for the separation of unsaturated from saturated fatty acid in general.[12]

[10] I wish to thank Drs. C. C. Hartman and W. T. Mitchell, Jr., for collecting the blood samples used in this work.

[11] Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

[12] To demonstrate how this method worked in my hands I give one of the preliminary analyses made of Merck's oleic acid labeled as highly purified. (A), 0.1700 gm. of oleic acid gave 0.1490 gm. of unsaturated fatty acids; yield 87.6 per cent. (B), 0.1080 gm. of oleic acid gave 0.0950 gm. of unsaturated fatty acids; yield 87.9 per cent. A third sample, where 0.1635 gm. of oleic acid was saponified first, gave 0.1558 gm. of unsaturated fatty acids; yield 95.3 per cent.

TABLE I.

Fatty Acids in 100 Cc. of Blood.

Case.	Sex.	Age.	Diagnosis.	Fat + lipoids.	Iodine No. on 100 gm. fat + lipoids.	Fatty acids.			Iodine No. on 100 gm. unsaturated fatty acids.	Hemoglobin (Sahli).	Red blood corpuscles.
						Total.	Unsaturated.				
		yrs.		*gm.*		*gm.*	*gm.*	*per cent of total*		*per cent*	
1. W. T. M.	M.	28	Normal.	0.790	73.7	0.345	0.168	48.8	84.2	98	5,520,000
2. F. A. C.	"	29	"	0.779	60.8	0.300	0.146	48.7	76.3	95	5,216,000
3. C. H. G.	"	30	"	0.755	67.2	0.300	0.136	45.5	92.1	105	6,984,000
4. C. P. B.	"	50	"	0.615	68.6	0.260	0.136	52.4	77.8	—	—
5. E. C.	"	21	"	0.619	—	0.313	0.136	43.4	105.9	—	—
6. K.	F.	24	"	0.627	59.8	0.263	0.137	52.0	88.8	90	4,200,000
7. O. S.	M.	28	Hemolytic jaundice 16 mos. after splenectomy.	0.839	62.0	0.240	0.137	57.3	142.4	63	3,200,000
8. L.	F.	41	Pernicious anemia.	—	—	0.286	0.160	55.9	94.8	15	680,000
9. S. M.	M.	46	" "	0.509	72.8	0.282	0.157	55.5	101.0	26	928,000
10. A. B.	"	29	Secondary anemia, jaundice.	0.564	75.3	0.241	0.141	58.5	107.8	35	3,690,000
11. McK.	"	56	" "	0.685	73.1	0.292	0.163	55.8	71.8	58	3,580,000
12. C.	"	17	Myelogenous leukemia.	0.355	90.1	0.290	0.140	48.3	110.9	15	800,000
13. S. M.	"	50	Cerebral hemorrhage.	0.955	57.9	0.453	0.318	70.2	92.8	105	5,640,000
14. O. H.	"	39	Chronic constipation.	0.777	88.7	0.362	0.255	70.4	111.5	100	5,008,000
15. N.	"	48	" nephritis.	0.890	78.6	0.370	0.252	68.2	136.6	68	4,440,000
16. F.	"	31	Myocardial insufficiency, chronic nephritis.	0.720	61.0	0.252	0.157	62.4	78.3	85	4,420 000
17. D.	"	30	Chronic nephritis.	0.818	67.3	0.333	0.154	46.3	89.8	94	4,328,000
18. H.	"	46	Diabetes mellitus.	0.699	75.9	0.307	0.173	56.5	86.0	90	5,040,000
19. S.	F.	58	" "	0.823	78.2	0.393	0.211	53.8	101.2	83	5,136,000

At the end of the titration of the fatty acids by the method of Gephart and Csonka[11] the fatty acids are present as potassium soaps in a mixture of petroleum ether and alcohol. A portion of the blood cholesterol is present in this same solution. To remove the cholesterol the solution is made alkaline, to insure separation more alcohol is added, and extracted twice with petroleum ether. The alkaline solution containing the fatty acids as soaps is transferred to a large beaker, evaporated on the water bath, the residue dissolved in warm distilled water, neutralized by acetic acid, and the fatty acids are precipitated as lead soaps by the addition of an excess of lead acetate solution. The soaps are then filtered off and dried in vacuum. The ether-soluble lead soaps are decomposed by the addition of hydrochloric acid; the free unsaturated fatty acids, after the ether is distilled off, are dried in vacuum, weighed, and saved for the determination of the iodine number according to Hübl.

Since the iodine number of the unsaturated fatty acids in several cases as shown in Table I is higher than that of oleic acid, the

TABLE II.

Case	3	9	10	14	18	19
Iodine No.	117.2	101.1	129.6	136.9	96.4	108.6
Unsaturated fatty acids of alcohol-soluble barium soaps, gm. in 100 cc. of blood	0.035	0.136	0.054	0.165	0.075	0.084

question of the direct evidence of the presence of other or higher unsaturated fatty acids arose in the latter part of this work. Therefore, the unsaturated fatty acids were dissolved in absolute alcohol neutralized by alcoholic KOH and the oleic acid was partly precipitated as barium oleate, placed in a refrigerator for a few hours, and then filtered. The unsaturated fatty acids of the alcohol-soluble barium soaps (Table II) were liberated by hydrochloric acid, extracted by ether, and after the ether was evaporated off the residue was dried, weighed, and used for the determination of the iodine number.

I was especially careful to avoid any alteration or destruction of the unsaturated fatty acids which may result from the use of excessive heat. The saponification and extraction flask described in the previous communication[9] was used to advantage in this work.

II.

A review of the literature has shown that in the determination of blood fats the extraction followed by saponification of the extract was to be preferred to the direct saponification. The former method was introduced by Shimidzu[13] who tried to apply the Kumagawa-Suto direct saponification, but found lower results than when he had first extracted the blood with alcohol and then saponified the extract. Berczeler[14] confirmed Shimidzu's claim and recently Rosenthal and Trowbridge[15] discussing the methods of fat determination concluded also that in blood Shimidzu's alcoholic extractions should precede saponification. As a control for the blood fat determinations in the present work it was thought advisable to run duplicate determinations according to the Gephart-Csonka[11] method. To saponify in alcoholic KOH medium

TABLE III.

Gm. of Fatty Acids in 100 Cc. of Blood.

Case	2	3	6	9	14	16	18
Extraction and saponification	0.300	0.300	0.263	0.282	0.362	0.252	0.307
Gephart-Csonka method	0.325	0.326	0.271	0.318	0.355	0.268	0.428

in a concentration sufficient to be sure that also the more resistant esters such as cholesterol esters are split with certainty, only 5 cc. of blood were used.

The results in Table III verify the applicability of the Gephart-Csonka method for blood in cases where we are interested only in the absolute values, with the advantage also of avoiding the time-consuming extraction. The above results show further that the accuracy was not limited by using such small amounts of blood; the higher results were expected, as the extraction is never complete, especially in case such large amounts of blood are extracted.

The method as applied to blood is as follows: To 5 cc. of blood measured into the author's saponification and extraction flask 20 cc. of alcohol

[13] Shimidzu, Y., *Biochem. Z.*, 1910, xxviii, 237.

[14] Berczeler, L., *Biochem. Z.*, 1912, xliv, 193.

[15] Rosenthal, H., and Trowbridge, P. F., *J. Biol. Chem.*, 1915, xx, 711.

(95 per cent) and 4 gm. of stick potassium hydroxide are added. The flask is immersed in a boiling water bath and its contents are boiled under a reflux condenser for 1 hour, it is then cooled, and 20 cc. of 20 per cent HCl are added in small portions to free the fatty acids, cooling the flask after each addition of the acid. The flask is filled to the constriction with distilled water, 50 cc. of ether are added, the flask is closed with the stopper, and shaken in a rotary manner for a few minutes. After the ether layer has separated it is blown by pressure into a separating funnel (250 cc.). The ether extraction is repeated twice with 50 cc. portions, collecting the portions in the separating funnel. After each extraction the ether layer is brought to the constriction by adding a little distilled water. The combined ether extract in the separating funnel is washed with several portions of water until the wash water is neutral. The crude ether extract, being free from hydrochloric acid as well as lower fatty acids, is transferred into an Erlenmeyer flask (200 cc.), a glass bead added, and the ether distilled off. The flask containing the residue is placed in the water bath for a few minutes, then in a vacuum desiccator over night to free the residue from moisture. 25 cc. of light, boiling petroleum ether are added, the flask is rotated at frequent intervals, and the solution filtered through a thick plug of fat-free cotton. Wash the flask and filter well with petroleum ether (3 times with 10 cc. portions) and collect the filtrate which is perfectly clear and colorless in an Erlenmeyer flask. Bring the petroleum ether solution to boiling in a water bath and titrate it immediately with 0.04 N alcoholic KOH using phenolphthalein as an indicator; each cc. of 0.04 N KOH is equal to 10.97 mg. of fatty acids. For further details see the original article.[11]

DISCUSSION.

The unsaturated fatty acids are of exogenous or endogenous origin, the latter being derived by synthesis from either carbohydrate or protein. A third source is by the desaturation of food fat and depot fat, which is mobilized for transport. Leathes[16] believes that desaturation is the rôle of the liver in fat metabolism, so we may consider that unsaturated fatty acids occur in normal metabolism.

As Table I shows, the blood of normal individuals contains an average of 0.143 gm. of unsaturated fatty acids per 100 cc. with an average iodine number of 87.5. 48 per cent of the total fatty acids are unsaturated. The iodine number of unsaturated fatty acids varies from 76 to 105 in normal human blood, thus indi-

[16] Leathes, J. B., *Ergebn. Physiol.*, 1909, viii, 356.

cating in addition to oleic acid the presence of other both higher and lower unsaturated fatty acids in small amounts.

In pathological conditions, we have found that a generally higher proportion of unsaturated fatty acids than the normal average occurs, and that the iodine absorption power of the unsaturated fatty acids as well as their absolute amount is higher. We may divide the pathological cases according to their hemoglobin content into two groups: (A) normal hemoglobin, and (B) low hemoglobin.

TABLE IV.

Group A.			Group B.		
Case.	Hemoglobin.	Iodine No.	Case.	Hemoglobin.	Iodine No.
	per cent			*per cent*	
13	105	92.8	7	63	142.4
14	100	111.5	8	15	94.8
16	85	78.3	9	26	101.0
17	94	89.8	10	35	107.8
18	90	86.0	11	58	71.8
19	83	101.2	12	15	110.9
			15	68	136.6

The high iodine numbers representing unsaturated fatty acids with higher degree of unsaturation than oleic acid are found in Group B, but we should not forget that in the same group we deal with an abnormally low red cell count. Munk and Friedenthal[17] found an increase of fat in red corpuscles during fat absorption, and Bloor[18] suggested that most, if not all, fat metabolized through lecithin, and that lecithin formation is a result of red cell activity. In anemia, where the number of red cells is lowered, probably the fat metabolism is disturbed; and as a fact we find the amount of lecithin as well as other lipoid substances generally lower than that in normal human blood. It would seem that these lipoid substances especially require the unsaturated fatty acids as a radical in their formation. The higher iodine value found especially in those cases where the lipoids are low suggests a resultant excess of unsaturated fatty acids and not necessarily an increased desaturation of fatty acids by stimulated liver activity.

[17] Munk, I., and Friedenthal, H., *Centr. Physiol.*, 1901–02, xv, 297

[18] Bloor, *J. Biol. Chem.*, 1916, xxiv, 447.

While we know that this excess of unsaturated fatty acids has a hemolytic action, and no doubt intensifies the hemolysis, yet it cannot be the primary cause, as we found in Cases 3, 13, 14, 19 (Tables I and II) higher unsaturated fatty acids in even larger quantities than in normal blood with normal red blood count and hemoglobin content.

The iodine values of fat plus lipoid substances show small variation in pathological cases as well as in normal cases, and have neither qualitative nor quantitative significance. A higher iodine number does not mean that there are present more highly unsaturated fatty acids than oleic, or that the proportion of unsaturated to saturated is larger.

SUMMARY.

Unsaturated fatty acids are a product of normal metabolism, being present in normal human blood, in which they form 48.0 per cent of the total fatty acids. Judged by their iodine absorption power there are, in addition to oleic acid, other unsaturated fatty acids both higher and lower than oleic, although in small amounts.

The iodine numbers of the unsaturated fatty acids in pathological conditions are generally higher than in normal individuals, especially in cases with low hemoglobin values. As such acids exist in normal human blood as well as in pathological conditions without anemia, it is necessary to look further for the primary cause of toxic hemolysis.

The Gephart-Csonka method for fatty acid determination in feces has been applied to blood in the present work, thus avoiding the time-consuming extraction common to other methods.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

V. THE NATURE OF THE DIETARY DEFICIENCIES OF A DIET DERIVED FROM PEAS, WHEAT FLOUR, AND COTTENSEED OIL.

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(Received for publication, January 25, 1918.)

There are described in the literature two types of experimental diets which are reported to have produced, the one in man and the other in dogs, a pathological condition which closely resembles, if indeed it is not actually identical with human pellagra (1, 2). It has long been suspected that pellagra is in some way the sequel to the long continued use of a faulty diet, but there have been various theories as to the manner in which the diet is at fault.

The experiments of Goldberger (1) have rendered the relationship of the diet to the disease extremely probable. Men were restricted during 5½ months to a diet prepared from wheat flour (patent), corn meal, (corn) grits, cornstarch, white polished rice, standard granulated sugar, cane syrup, sweet potatoes, pork fat (fried out of salt pork), cabbage collards, turnip greens, and coffee. At the end of 5½ months, five of the eleven men thus restricted, showed unmistakable signs of the disease. Goldberger interpreted his results with creditable caution, but decided that, "On the whole, however, the trend of available evidence, strongly suggests that pellagra will prove to be a 'deficiency' disease very closely related to beri-beri" (1).

Voegtlin (3), after a critical examination of the literature, and also from clinical observations, formulated his views concerning the etiology of pellagra as follows:

"From a survey of the clinical and pathological aspects of pellagra, I have arrived at the conclusion that we are dealing with a chronic intoxication. While the agents at work in this intoxication are as yet unknown, I am inclined to believe that toxic substances exist in certain vegetable food, not necessarily spoiled, which if consumed by man over a long period

of time, may produce an injurious effect on certain organs of the body. This hypothesis does not rule out the possibility that a dietary deficiency . . . . [vitamines] . . . . may play a rôle in the production and treatment of pellagra."

"One will have to consider very seriously: (1) A deficiency or absence of certain vitamines in the diet. (2) The toxic effect of some substances, as aluminum, which occur in certain vegetable foods. (3) A deficiency of the diet in certain amino-acids."

Chittenden and Underhill (2) produced in dogs a condition which they describe as closely similar to pellagra in man, by feeding a diet consisting of cooked (dried) peas, cracker meal, and cottonseed oil. Their views concerning the number of diseases which fall into the same category with beri-beri, that is in being caused by the lack of specific protective substances, are formulated as follows:

"The absence or deficiency of these substances ('vitamines') may lead to a variety of metabolic disturbances which have been designated by different names as beri-beri, scurvy, pellagra, etc., and which may be grouped together as 'deficiency diseases.'" They concluded from their numerous experimental data that, "From the facts enumerated the conclusion seems tenable that the abnormal state may be referred to a deficiency of some essential dietary constituent or constituents, presumably belonging to the group of hitherto unrecognized but essential components of an adequate diet."

As a result of studies with diets composed of purified foodstuffs supplemented with fats of the growth-promoting and non-growth-promoting classes, and with alcoholic and water extracts of plant tissues (4) and also with the addition of small amounts of various natural foods (5), we were led to the conclusion that there are possible of classification two still unidentified food complexes which are essential in the diet, and we introduced the terms fat-soluble A and water-soluble B to designate them (6). The prefixes "fat-soluble" and "water-soluble" have now become generally adopted by other investigators, although there is still lack of uniformity in the terminology in other respects. There are not less than twenty different terms in the literature of the last 2 or 3 years to designate these two substances, or as a few authors seem to believe, groups of substances of unknown nature.[1]

[1] Recently Emmett and McKim (7) have argued in favor of the existence of a special "vitamine" for maintenance and another or others for growth. We are not able to see in the experimental data which Emmett and McKim present any evidence in support of the interpretation which the authors put upon their data.

It is obviously of the greatest importance that we have a correct understanding of the relation of diet to disease, and it is accordingly exceedingly desirable that it be definitely established whether there is in reality but a single essential substance in each of what we term fat-soluble A and water-soluble B. If, as the evidence available indicates, each represents but a single substance, there can be but two deficiency diseases in the sense in which Funk employed this term (8). He used the term "deficiency diseases" to designate a series of four or more syndromes, beri-beri, scurvy, pellagra, and rickets, and expressed the belief that each was due to the lack of a specific protective substance. We have, therefore, directed our efforts to the solution of the problem concerning the number of the unidentified dietary essentials, and have repeatedly expressed the view that there are but two (9).

McCollum and Pitz (10) demonstrated that scurvy in the guinea pig does not belong in the category of "deficiency" diseases, and referred it instead to the agency of microorganisms in the digestive tract. The stagnation of feces in the cecum of the guinea pigs employed as experimental animals was suggested to be the primary cause of the development of the organisms concerned. Hess (11) has recently described experiments which support our conclusions regarding the bacteriological factor in the production of scurvy. He found that freshly pasteurized milk does not produce scurvy in the human infant, whereas the same milk kept 24 hours after the heat treatment may do so. The most plausible explanation for this is the development of pernicious forms of organisms after the pasteurization has checked the lactic acid formers. We hold, therefore, that scurvy has been definitely eliminated from the list of supposed "deficiency" diseases.

In the previous articles of this series, we have analyzed the deficiencies of several seeds, from the dietary standpoint, by suitably planned feeding experiments (12, 13). We have arrived at the conclusion that beri-beri was properly judged by Funk as to the cause of its origin, but that the other diseases which he held to be due to the lack of specific substances from the diet are in reality due to other causes. We have called attention to clinical evidence of the existence in man of cases of xerophthalmia (9), entirely analogous in origin to the same pathological state in rats, brought about by a deficiency of the fat-soluble A in the diet. Xerophthalmia and polyneuritis are abundantly demon-

strated to have their origin in the lack of a sufficient amount of the fat-soluble A and water-soluble B respectively in the diet. The experiments described in this paper make it clear that the diet of Chittenden and Underhill is not deficient in the sense that it fails to furnish a sufficient amount of another specific substance which when present protects against the development of the syndrome of pellagra. The deficiencies of their diet are all dependent upon the shortage of the fat-soluble A, the character of the inorganic moiety, and the relatively poor quality of its protein mixture. The experimental demonstration of this fact, provided the interpretation be accepted that their dogs were suffering from a disease analogous to pellagra in man, eliminates a second syndrome, pellagra, from the list of supposed "deficiency" diseases.

On the appearance of Chittenden and Underhill's paper, we were at once convinced that their interpretation of the nature of the deficiencies of their diet could be only partially correct. This conclusion is inevitable in the light of our many studies of all the more important seeds, which are in use as foods for men and animals. Each of these, we have shown, can be supplemented by the addition of inorganic salts, purified protein, and a growth-promoting fat, so as to be dietetically complete (14). It must follow, therefore, that the fat-soluble A is the only unidentified dietary factor of which there is any relative lack in any of the seeds which we have studied. The deficiency of the seeds in fat-soluble A is only relative, not absolute. Each of the seeds appears to contain at least 50 per cent of the requirements of the growing rat for this substance, provided the other factors in the diet are of good quality. A mixture of peas, wheat flour (or crackers), and cottonseed oil, should, in the light of our data with each of the common seeds, be susceptible of fairly accurate appraisal as to its dietetic value. The experiments described in this paper demonstrate the correctness of this view.

Chart 1. Lot 1,915.—The standard food mixtures of peas, patent flour, and cottonseed oil induced no growth in Period 1. In Period 2 the addition of purified protein did not correct the ration so as to produce growth.

Lot 1,916 A shows that a suitable salt addition was not sufficient to render this food mixture capable of supporting growth,

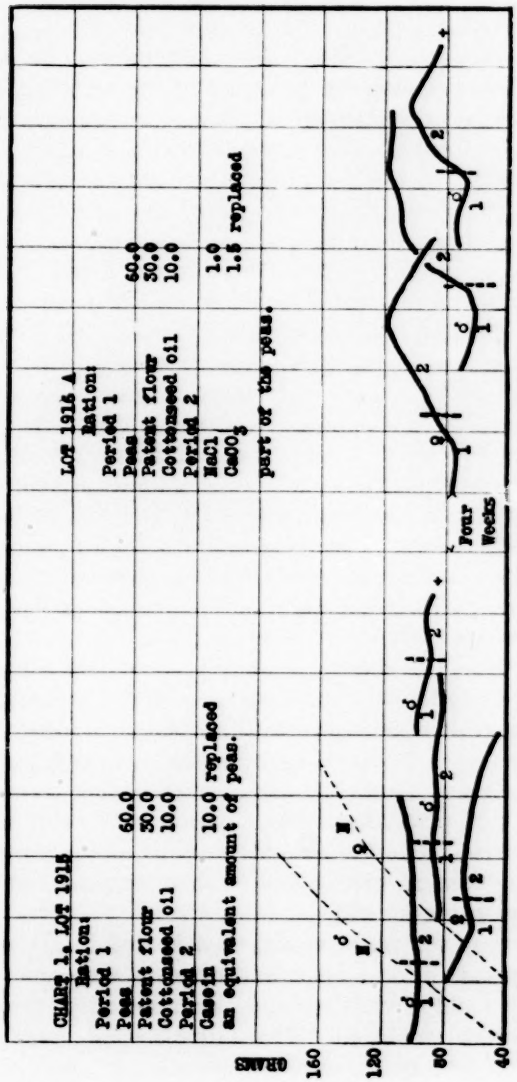
CHART 1. LOT 1918
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
Casein 10.0 replaced
an equivalent amount of peas.
LOT 1918 A
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
NaCl 1.0
CaCO3 1.5 replaced
part of the peas.
GRAMS
160
120
80
40
Four Weeks

CHART 1.

while Lot 1,021, Chart 2, and Lot 1,022, Chart 3, demonstrate that the content of the dietary factor, fat-soluble A, is not the factor limiting growth. No matter how well the proteins of this diet are supplemented with purified protein addition, with or without increasing the content of the diet in fat-soluble A, growth cannot take place unless the elements sodium, chlorine, and calcium are added. We have shown in the first (12) and fourth papers (15) of this series that these are the only inorganic elements which are required to be added to any of the seeds in order to make them dietetically complete, as far as the mineral content is concerned. Compare Chart 1 with Charts 2 and 3.

These groups of rats developed very rough scaly tails, and numerous bleeding points covered the surface. The ears were thickened and on the margins scabs developed. There was a cutaneous horn on the nose of each rat. These signs of pathological changes on the skin are common in our rat colony in animals fed certain types of faulty diets. They are not the unfailing accompaniment of diets which cause debility even of a severe character. We have not yet analyzed with sufficient thoroughness the exact nature of dietary faults which induce these changes.

There was no diarrhea in any of our rats on the diets described in this paper. Neither did we observe infection in the oral and intestinal mucosa.

Lot 1,916 A.—The diet in Period 1 could not support any growth. In Period 2, they received in addition sodium chloride and calcium carbonate. The salt additions induced a slight response with growth for 6 to 8 weeks, followed by decline. Lot 1,021, Chart 2, shows the necessity of adding these salts, if growth is to take place; Lot 1,023, Chart 3, shows that no others are necessary to enable the animals to grow at the normal rate. The inorganic content of this diet is, therefore, the first limiting factor in preventing growth.

The peas were soaked in water, then heated in a sterilizer at 15 pounds' pressure for 1 hour, and dried in a current of air at about 70°C. Gold Medal flour and a commercially bleached cottonseed oil were employed. The animals were given distilled water, and according to our general custom, were furnished water containing iodine in potassium iodide once each week.

Chart 2. Lot 1,916 B.—In Period 1 these rats received the

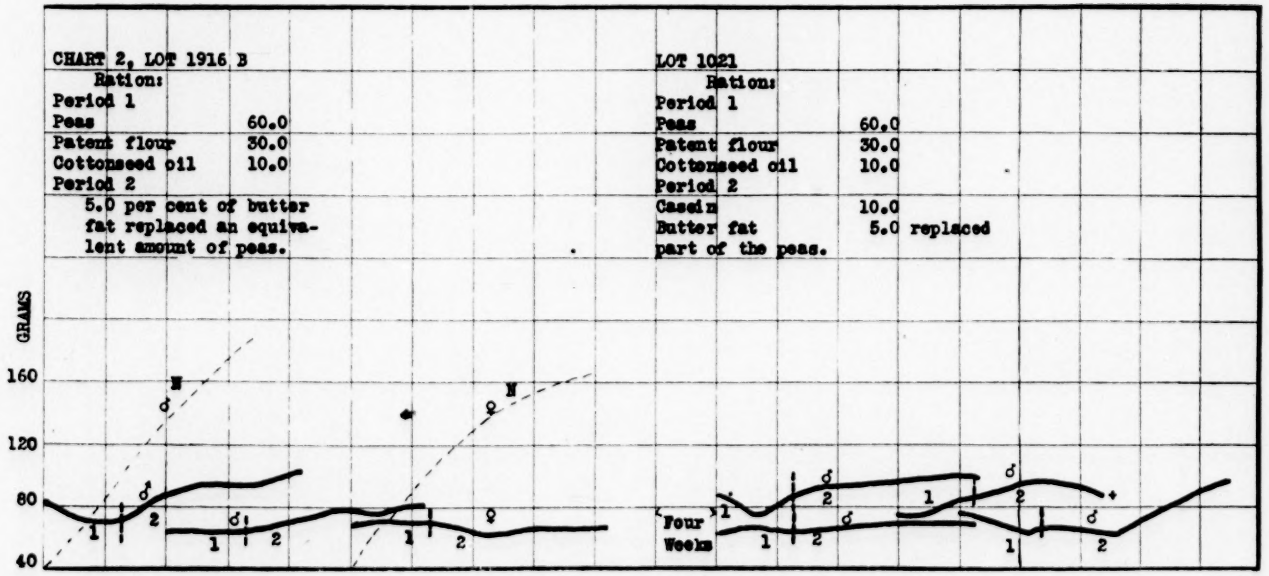
CHART 2, LOT 1916 B
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
5.0 per cent of butter fat replaced an equivalent amount of peas.
LOT 1021
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
Casein 10.0
Butter fat 5.0 replaced part of the peas.
GRAMS
160
120
80
40
Four Weeks

CHART 2.

standard mixture and failed to grow. In Period 2 the content of the fat-soluble A was increased by the addition of butter fat, but without noticeable benefit. Chart 1, Lot 1,916 A, showed that the addition of an appropriate inorganic salt mixture, and Lot 1,915 an addition of purified protein did not supplement the mixture of peas, patent flour, and cottonseed oil so as to induce growth. Two purified food additions, one of which is inorganic, are necessary (Chart 3, Lots 1,022 and 1,023). These rats (Lot 1,916 B) showed the same skin lesions on the nose, ears, and tails that were described in Chart 1, Lot 1,915. They differed from the latter in being extraordinarily irritable. They were terror stricken whenever the cage door was opened, and there was always great difficulty in weighing them. Irritability in some degree was characteristic of all the rats on this ration where but one purified food addition was made. Extreme timidity is of common occurrence in our experimental rats on certain types of faulty diet. We are not yet in a position to discuss satisfactorily the exact nature of the causes of this abnormality.

Lot 1,021.—These records illustrate the fact, when interpreted together with Chart 3, that both protein and fat-soluble A (as butter fat) added to the diet of Period 1 fail to make the ration capable of supporting growth. The addition of certain salts (sodium, chlorine, and calcium) is necessary before growth can proceed. These salts alone are, however, not sufficient (Chart 1, Lot 1,916 A). Either the protein must be improved, or the fat-soluble A must be increased in amount, as well as an improvement in the inorganic moiety effected before the food mixture becomes capable of supporting growth (Chart 3, Lots 1,022 and 1,023). It is not apparent from the curves of growth that it is of great importance for some weeks whether the second factor which is improved is protein or fat-soluble A. Growth can take place in either case. Prolonged well-being requires, however, in addition to other supplements, an increase in the content of fat-soluble A in this food mixture.

Chart 3. Lot 1,022.—In Period 1 the animals were fed the standard mixture of peas, patent flour, and cottonseed oil and were unable to grow. In Period 2, the addition of two purified food substances, the simple salt mixture of sodium chloride and calcium carbonate, together with purified casein, induced prompt

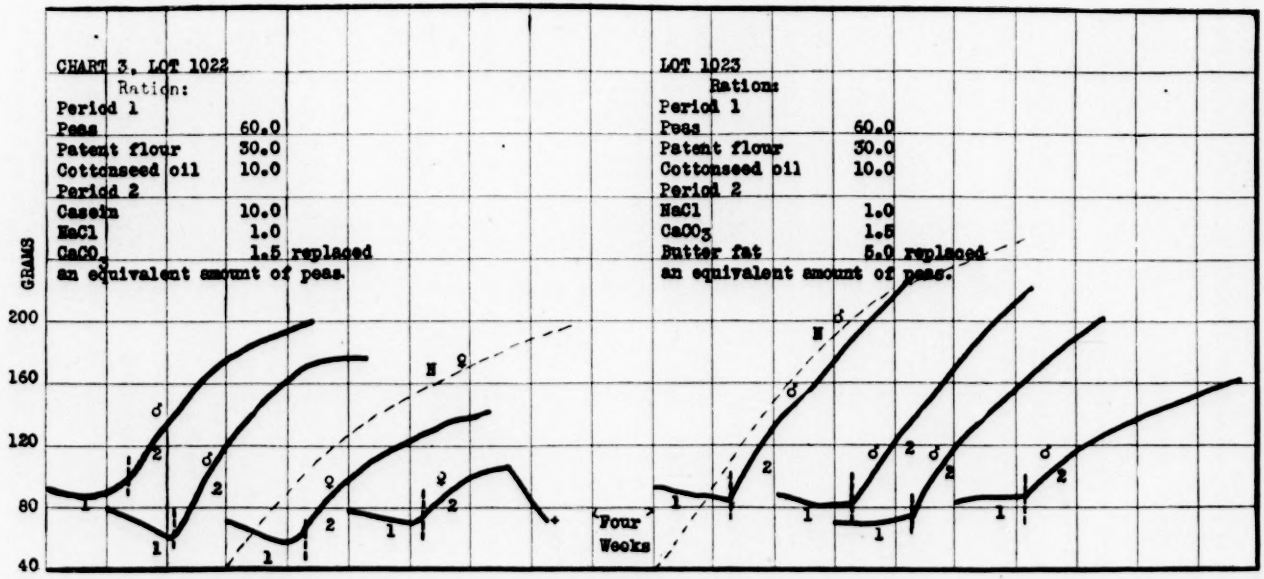
CHART 3. LOT 1022
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
Casein 10.0
NaCl 1.0
CaCO3 1.5 replaced
an equivalent amount of peas.
LOT 1023
Ration:
Period 1
Peas 60.0
Patent flour 30.0
Cottonseed oil 10.0
Period 2
NaCl 1.0
CaCO3 1.5
Butter fat 5.0 replaced
an equivalent amount of peas.
GRAMS
200
160
120
80
40
Four Weeks

Chart 3.

response. Chart 2, Lot 1,021, showed that two purified food additions, protein and fat-soluble A, do not enhance the diet so as to support growth.

The growth of these animals was not so rapid as they are capable of making when the diet is highly satisfactory. This is illustrated by the records of Lot 1,024, Chart 4, when compared with Lots 1,022 and 1,023, Chart 3. In the latter the best rats became stunted before reaching full adult size. These records make it clear that the diet of peas, wheat flour, and cottonseed oil can be supplemented with well known dietary components so as to induce a moderate amount of growth in the rat. It is necessary to conclude, therefore, that the dietary deficiencies of this food mixture, which has induced in dogs symptoms closely resembling pellagra in man, are not in the nature of unidentified "protective" substances, except that there is in some degree a shortage of the content of fat-soluble A. Sodium chloride and calcium carbonate, together with purified protein and fat-soluble A, render the diet practically complete (Chart 4).

Lot 1,023.—These records illustrate the fact that the protein mixture derived from peas and patent flour (12.1 per cent) is adequate for the support of a good rate of growth (Period 2) after a period of stunting for 5 weeks. These records further support the view that the first limiting factor in this food mixture is the quality of the inorganic content (see Charts 1 and 2). These growth curves are not of so steep a gradient, however, as can be secured by the further improvement of the protein content of the diet by the addition of purified casein (Chart 4). A comparison of Charts 3 and 4 makes it evident that more fat-soluble A must be added before the optimum growth can be secured with this ration.

Chart 4. Lot 1,024.—These records show the degree of improvement of a mixture of peas, patent flour, and cottonseed oil, which can be effected by the addition of three recognized dietary factors, protein, salts, and fat-soluble A. Not only is the growth on the diet supplemented in this way nearly at the maximum rate, but three litters of young have been secured.

A mixture of peas, patent flour (crackers), and cottonseed oil induced in dogs a profound state of malnutrition, characterized by sore mouth, sloughing of the mucosa, diarrhea, infection of the

mucosa of the intestinal tract, and skin lesions (2). The same diet is shown in this paper to be deficient only with respect to three dietary factors, all of which can be named and but one of which (fat-soluble A) is of chemically unknown nature. This forms a strong argument against the idea that pellagra is a disease in the same category with beri-beri, or the xerophthalmia which was described in the second paper of this series (9). Pel-

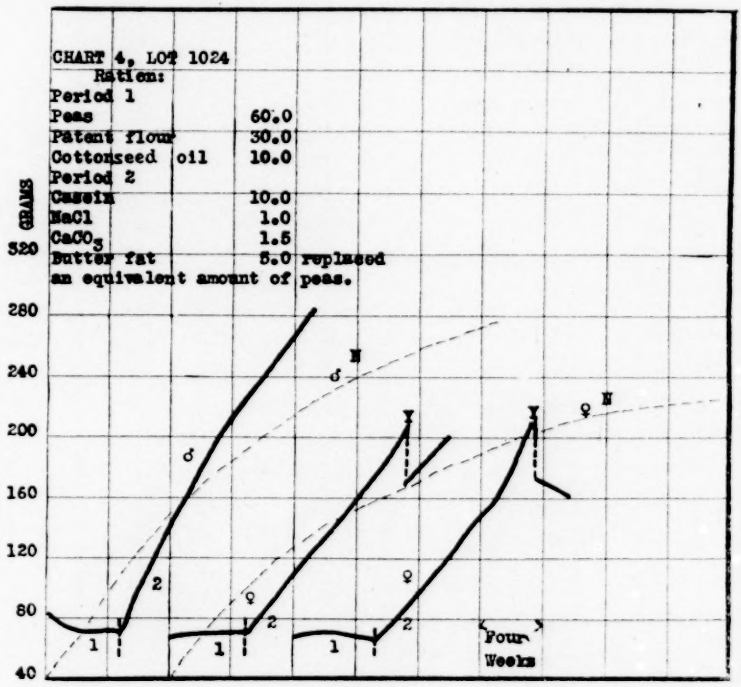

CHART 4.

lagra is primarily, we believe, associated with the unsatisfactory character of three dietary factors as described, and there cannot be a specific protective substance against this syndrome as there is for the other diseases just enumerated. We have elsewhere offered chemical evidence that there is but one water-soluble indispensable food constituent of unknown chemical nature (13). The demonstration that neither scurvy nor pellagra belong in

the class of disease for which such protective substances exist, removes the necessity of postulating the existence of more than one indispensable substance in what we term water-soluble B, unless it shall be shown that rickets, the only other disease referable to faulty diet, is comparable in its etiology with beri-beri and xerophthalmia. The experiments reported in this paper support our contention that there are in reality but two "de-

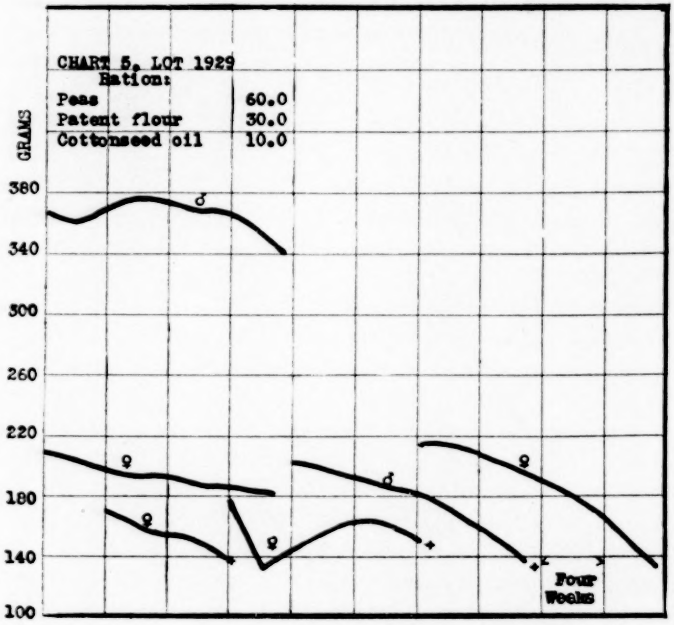

CHART 5.

ficiency" diseases, and but two physiologically indispensable unidentified dietary essentials.

Chart 5. Lot 1,929.—In this chart are shown for comparison with the growth curves of young animals (Charts 1 to 4 inclusive) the curves illustrating the changes in body weight of grown rats confined to the diet of peas, flour, and cottonseed oil. These were at the beginning of the experiment about 1 year old and in excellent condition, and capable, if fed a good ration, of living about 2 years longer. They were restricted to the diet of peas.

patent flour, and cottonseed oil, which was employed throughout this work. All suffered steady decline, showing that the diet is not of a character to support either maintenance or growth until properly supplemented. Three of the six animals died at the end of the 8th, 12th, and 15th weeks respectively.

The coats of these animals became rough but there were no thickening of the ears, baldness, soreness of the tails, or cutaneous horns on the noses, such as we have seen to be concomitant with faulty diet. We have not observed therefore, the pathological changes in the mucous surfaces of the alimentary tract nor the skin changes and infections observed by Chittenden and Underhill in dogs. In grown rats, loss of weight and the early appearance of senile characters were the only evidences that this diet was inadequate. In certain groups (Charts 1 and 2) in young rats skin changes were observed.

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THE STRUCTURE OF YEAST NUCLEIC ACID.

III. AMMONIA HYDROLYSIS.

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(Received for publication, January 31, 1918.)

Since the tetranucleotide structure of yeast nucleic acid has been generally accepted, the efforts of workers in this field of investigation have been devoted to a search for an explanation of the mode of linkage between individual nucleotides. The theory of the structure of yeast nucleic acid was established through the discovery of methods which yielded on the one hand the individual nucleotides and on the other the pyrimidine nucleotides. For the explanation of the further details in the structure of the substance it was necessary to obtain a fragment of the nucleic acid molecule that would possess a more complex structure than the simple mononucleotides. Attempts in this direction were made by Thannhauser and Dorfmüller[1] and by Walter Jones[2,3,4] and his coworkers. Thannhauser and Dorfmüller reported the discovery of a trinucleotide, Walter Jones with his coworkers the discovery of two dinucleotides. The conclusions of Thannhauser and Dorfmüller were criticised by Jones who very convincingly exposed the weak points in the arguments of Thannhauser.

However, it is now found that the claim of Jones and Germann and Jones and Read to have isolated an adenine-uracil dinucleotide was not well founded. Following exactly the same conditions of analysis as given by these authors a brucine salt was obtained which possessed analytical values required by the dinucleotide. On recrystallization out of 35 per cent ethyl alcohol this brucine

[1] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1915, xcv, 259.

[2] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

[3] Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

[4] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123; xxxi, 39.

salt was separated into two fractions, one analyzing for uridinephosphoric acid, the other for adenosinephosphoric acid. Out of 120 gm. of the mixed salt there were obtained 50 gm. of the first portion and 70.0 gm. of the second. The first salt was converted into a barium salt which possessed the crystal form, the optical rotation, and the analytical values of the recently described[5] barium salt of uridinephosphoric acid.

The second fraction was also converted into a barium salt which separated out of a concentrated aqueous solution. However, it was impossible thus far to obtain the salt in crystalline form. Work in this direction is in progress.

It is evident on the basis of this experience that as far as the yeast nucleic acid is concerned the largest fragment obtained up to the present is a mononucleotide. Hence the problem of the mode of linkage between the individual nucleotides still awaits its solution. On the other hand the work done in Jones' laboratory and our recent work have advanced further proof for the tetranucleotide structure of yeast nucleic acid, since on cleavage of the nucleic acid it is now possible to isolate the following three mononucleotides in pure form: guanylic acid, uridinephosphoric acid, and cytidinephosphoric acid; and there is reasonable hope that adenosinephosphoric acid also will be prepared in pure form.

EXPERIMENTAL.

Crude nucleic acid in lots of 100.0 gm. each in 500 cc. of water and 50 cc. of 25 per cent ammonia water were hydrolyzed for 1 hour in an autoclave at 115°C. The reaction product was filtered and to the filtrate an equal volume of 98 per cent alcohol was added. A precipitate was thus formed which was removed by filtration. The filtrate was concentrated to a third of the original volume under diminished pressure (between 12 and 15 mm.), the temperature of the water bath not exceeding 40°C. To the concentrated solution was added again an equal volume of 98 per cent alcohol. To the filtrate a 25 per cent solution of basic lead acetate was added as long as a precipitate formed.

The lead precipitate was ground up in a solution containing 5 per cent lead acetate and filtered. The operation was repeated three times. The washed lead precipitate was suspended in water

[5] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 229.

containing barium hydroxide and decomposed by means of hydrogen sulfide. Care was taken to keep the solution slightly on the alkaline side. The excess of hydrogen sulfide was removed by aeration; the solution was then made distinctly alkaline, filtered, again neutralized, and concentrated to a volume of 500 cc. The barium was then removed quantitatively and from the solution the brucine salts were prepared in the usual way.

The analysis of the mixed brucine salts gave the following value.

0.200 gm. substance gave 15.4 cc. N at 18°C. and 747.2 mm.

For cytosine-uracil dinucleotide $C_{19}H_{25}N_7P_2O_{15}.4(C_{23}H_{26}N_2O_4)+14\ H_2O$

	Calculated:	Found:
N	8.46	8.88

This material was recrystallized nine times. The final product gave the following analytical results.

0.200 gm. substance gave 12.4 cc. N at 17°C. and 757.2 mm.
0.1012 " " " 0.1970 gm. CO_2, 0.0582 gm. H_2O, and 0.0058 gm. ash.

	Calculated for $C_{23}H_{26}N_2PO_7+7H_2O$:	Found:
C	53.20	53.08
H	6.51	6.43
N	6.80	7.26

This brucine salt was then converted into the barium salt. The salt crystallized in the manner described in the previous communication. It was redissolved in a small volume of a 10 per cent solution of sulfuric acid. The solution was neutralized with barium hydroxide, filtered, and allowed to crystallize at room temperature. The figure illustrates the crystals.

The substance gave the following analytical data.

0.200 gm. substance required for neutralization 8.92 cc. of 0.1 N acid.
0.1052 " " gave 0.0912 gm. CO_2, 0.0230 gm. H_2O, and 0.0514 gm. ash.

	Calculated for $C_5H_{11}N_2O_9PBa$:	Found:
C	23.50	23.64
H	2.41	2.45
N	6.10	6.24
$Ba_2P_2O_7$	48.97	48.86

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+0.14^\circ \times 100}{1 \times 4} = +3.5^\circ.$$

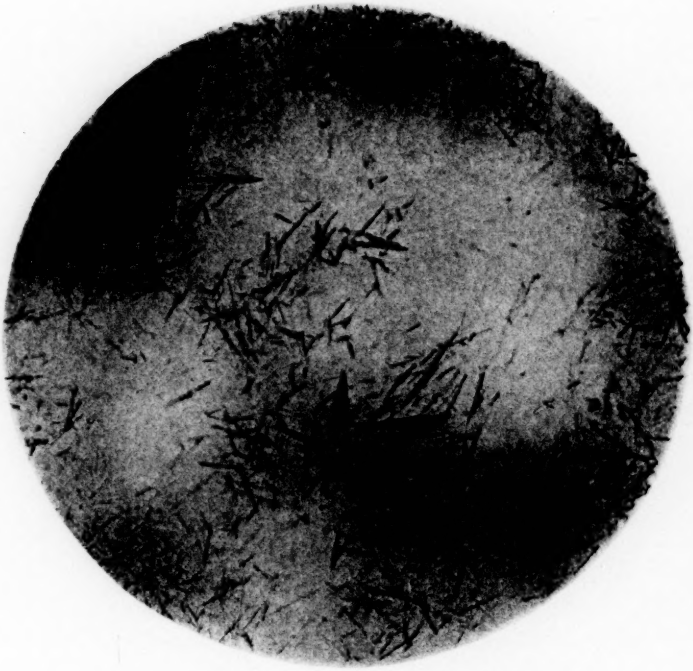

FIG. 1. Barium salt of uridinephosphoric acid.

A DETAILED METHOD FOR THE PREPARATION OF HISTIDINE.

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(From the Psychopathic Laboratory of Cook County Hospital, Chicago.)

(Received for publication, January 18, 1918.)

The many uses which are being found for histidine in various lines of biological work and the difficulty of obtaining this amino-acid on the market make it necessary for many research laboratories to prepare their own supplies of histidine.

Directions for the preparation of histidine as given in the text-books are usually too meager to lead one to a successful first trial; *i.e.*, a good yield and a pure product. Either a large amount of the product is discarded unawares in one of the precipitates or filtrates, or else the mother liquor is too grossly contaminated with other salts to permit the product to separate in pure crystals. The method given here is offered, however, not as an improvement on the method already in use, but as a more detailed statement of that method, together with reasons for certain apparently insignificant steps in the process which are easily overlooked.

Hemoglobin is the protein of preference as a source of histidine, and ox blood corpuscles, or the so called blood paste,[1] which can be obtained from the slaughter house, are probably the most economical source of histidine.

2 liters of blood paste are placed in a wide-bottomed dish or porcelain-lined pan and to this is added slowly an equal quantity of concentrated HCl. The lumping of the corpuscles into large masses is of no significance, as these lumps will dissolve later. The mixture is then poured into an 8 or 10 liter Jena or Pyrex round-bottomed flask and heated on a sand bath in the fume chamber for about 18 hours. By this time the volume of the mixture will be reduced to about half the original volume; *i.e.*, 2 liters. The biuret test on a neutralized and filtered portion should now be negative. If the biuret test is negative, the flame

[1] "Blood paste" is the concentrated suspension of red blood corpuscles obtained by centrifugating defibrinated ox blood.

is removed and the mixture allowed to cool. It is then mixed with an equal volume of saturated sodium carbonate, pouring the acid mixture slowly into the carbonate solution, with thorough mixing. The vessel for this should have a capacity of 40 or 50 liters to allow for the effervescence of such a viscous mass. Finally, after the foam has settled, which requires a few hours, the mixture should be made exactly neutral to litmus paper and filtered. If it has not been carefully neutralized, this filtrate will have a reddish purple muddy color instead of a clear yellow. The filtrate is now made alkaline with 25 gm. of NaOH and boiled to expel ammonia. After an hour of boiling, it may be necessary to add more NaOH to make sure that all the ammonia will be driven off. Any NH_4Cl left in the solution will interfere in all subsequent treatments of the precipitates and filtrates, and will eventually have to be separated from the final product.

After standing for several hours in the cold, the leucine and tyrosine will separate out, and should be filtered off. The filtrate should then be treated, alternately, with concentrated solutions of $HgCl_2$ and of Na_2CO_3, using the Na_2CO_3 to keep the mixture alkaline as more $HgCl_2$ is added. Continue this until a small filtered portion, which should be distinctly alkaline to phenolphthalein, does not produce the heavy voluminous precipitate of mercuric histidine when treated with more of the $HgCl_2$ solution. An opalescent or milky precipitate will continue to form even after all the histidine is precipitated, but should be disregarded. Now filter off the large volume of precipitate and wash by suspending the latter in a 2 liter quantity of distilled water and again filtering. This process is repeated a second time to insure the removal of substances in the original solution, as well as the excess of $HgCl_2$. Suspend the twice washed precipitate in five times its volume of water and, using concentrated HCl, render the latter acid to bromophenol blue (pH = 3). If the dilution is greater than five volumes of water, the same volume of concentrated HCl would be required, but the pH would be higher; and since both the dilution and the pH are more easily measured than the strength of the acid and the volume of precipitate, this method is suggested. At such a dilution, and pH, the mercuric histidine will be redissolved, while a large volume of a dirty brown residue should remain in suspension. The latter is then removed by

filtering. The acid filtrate is then treated with a sufficient quantity of concentrated solution of Na_2CO_3 to render the solution distinctly alkaline to phenolphthalein, whereupon the mercuric histidine is reprecipitated as a clean, creamy flocculent mass. The latter is thoroughly washed two or three times by suspending in water and refiltering as before, in order to remove Na_2CO_3 and NaCl.

Finally, it should be suspended in about 600 cc. of water, and H_2S allowed to bubble slowly through the thick suspension, until the mass becomes coal-black. The filtrate from this mass of HgS is then allowed to evaporate at room temperature, whereupon large yellowish crystals of histidine hydrochloride will separate out from the brown, syrupy mother liquor. The yield should be about 15 gm. The crystals are then freed from the brown adhering film of mother liquor, and dried between filters. It should be identified by its melting point (256°), by its crystalline structure, and i s freedom from inorganic salts, proved by incinerating a small portion on platinum foil.

THE GROWTH OF CHICKENS IN CONFINEMENT.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

Plates 4 to 6.

(Received for publication, January 10, 1918.)

The possible advantage in the use of chickens as experimental animals in the study of growth and other problems of nutrition has suggested itself to many investigators since the days of Pasteur. These birds can be hatched under artificial conditions; they grow rapidly and therefore may furnish desired evidence more speedily than is the case with most of the domestic animals; and furthermore if the conditions under which chickens continue to grow normally in confinement can be learned, it will be possible to obtain much information of practical use in poultry husbandry.

There is a widespread belief among poultry raisers that young chickens cannot be reared under the artificial conditions of housing and diet which many other experimental animals tolerate without detriment. The current ideas are expressed in the statements that the birds must be kept "on the ground," that they must have exercise, outdoor life, and green food. In a recent report of observations upon the growth of young chickens under laboratory conditions Drummond[1] wrote:

> "There are not many published records of the use of young chickens as the experimental animals in studies upon the factors concerned in growth and nutrition, but, from a reference to those of recent date, the present author was led to believe that one might rely to some considerable extent upon their suitability for such studies."

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Drummond, J. C., *Biochem. J.*, 1916, x, 77.

Drummond attempted to rear young chickens in the laboratory without success even when the food supplied was supposed to be the same as that which facilitated growth under conditions of normal environment. He mentions "ruffled appearance" and "weakness in the legs" as symptoms attending the failures. Quoting Drummond further:

"The failure of the normal birds to grow and develop was at first attributed to possible defects in the condition of keeping or feeding. Thus, poultry-keepers sometimes consider that weakness in the legs is due to a deficiency of fresh meat in the diet. It was therefore attempted to rear a batch of young chicks upon normal food, giving them in addition a ration of fresh meat daily. The birds ate the meat greedily, but did not respond to the change, and the effort to raise them was no more successful than before.

It was then thought that if the young birds were reared upon a more natural surface they might thrive more satisfactorily. Accordingly, two batches were reared separately, one being brought up upon a surface of sawdust and coarse sand, scattered on the floor of the cage . . . whilst the other group was provided with a surface of fresh earth and grass."

Post-mortem examination of a large number of these 'normal' chicks did not bring to my notice any particular point of interest. The bodies were without exception greatly emaciated. The organs were usually small and anaemic. During life this apparent poverty in blood was striking. The combs and eyelids of the chicks were peculiarly bloodless, being of a dead white colour, as contrasted with the deep red colour possessed by the combs and eyelids of normal fowls of an equal age."

In conclusion Drummond wrote:

"It is felt that the young chick is not a suitable object upon which to conduct experiments in the study of growth, at any rate, when it is kept under artificial conditions. The difficulty of rearing young fowls indoors is, I believe, realised by poultry-farmers generally, so much so in fact, that it is seldom attempted by them.

An explanation of the cause of the failure has not yet suggested itself. No dietary deficiency can be held as responsible, for the diet supplied to the experimental chicks in the normal batches was in every way similar to that successfully employed to rear chicks in the open air. An attempt to supply one of the conditions, under which fowls are normally reared, was made when fresh earth and grass were given to provide a surface for the young birds to run upon.

They certainly made use of the soil, scratching it over and picking out small insects and other tasty morsels all day long. And yet, despite the fact that the earth was changed frequently, this group of birds reached but a slightly better standard of development than did a similar batch, which had a surface of sawdust and grit to run upon.

In the ight of these facts we must admit that there are other factors than an adequate diet, which must be supplied to the growing chick, in order that it may complete its normal growth when under conditions such as are described."

Likewise Funk,[2] in referring to Drummond's conclusion, that even chickens on normal diet do not thrive when kept under laboratory conditions, wrote:

"This fact, of which I am fully aware, has been already emphasized in one of our publications. . . . . Still it also remains a fact that as a result of an inquiry into conditions existing in poultry farms, we are using now a mixture of chicken food, cabbage, and charcoal, the water being entirely replaced by milk, thereby succeeding in diminishing the mortality to a very great extent, in spite of the fact that the birds are kept in small cages."

The experience which we[3] reported in feeding young chicks on selected rations encouraged us to make further attempts to ascertain the conditions under which these birds can be used, if possible, in the laboratory for the study of the factors essential for their nutrition during growth. Two of the chickens, Nos. 5 ♂ and 6 ♂, reported in our earlier paper at the age of 81 days, subsequently attained maximum body weights of over 6 pounds—2,760 and 2,915 gm. respectively at the age of 309 days. Their plumage was well developed, their appearance excellent, and both proved fertile. During a period of almost a year they were kept in cages not over 2 x 2 x 2 feet, were never removed from the laboratory, and never received any green fodder. Photographs taken at the age of 322 days are appended.

During the period of these experiments the protein concentrates fed were corn gluten, cottonseed flour, soy bean flour, and casein. "Protein-free milk" and butter fat were used as sources of "vitamines." Starch, lard, and ferric citrate also entered into the diet. An abundance of water and grit was always furnished.

Since these rations contained no roughage we early trained these chicks to eat the blotting paper with which the bottoms of their cages were lined. The importance of an adequate supply of roughage seems to have been overlooked by those who have at-

[2] Funk, C., *J. Biol. Chem.*, 1916, xxvii, 3, footnote.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 293.

tempted to raise chickens in confinement; and the good effects which appeared to result from the use of paper fiber have led us to continue to feed it in the form of blotting paper or moist paper pulp in our subsequent experiments with chickens. It is surprising with what avidity large quantities of paper were eaten, full grown birds consuming daily a sheet of blotting paper nearly 2 feet square. By supplying this cellulose material the use of the more complex forms of roughage, as grass, straw, etc., was avoided. It is possible that the employment of plenty of oat straw litter by poultrymen has its advantage in the supply of roughage thus furnished rather than in the exercise which it is generally supposed to stimulate.

Out of ten 3 weeks old White Leghorn chicks similarly put on various experimental rations and deprived of all green food, four attained a considerable size. Photographs of two of these are shown at an age of 271 days and weights of 1,520 gm. (3.3 pounds, cockerel) and 1,130 gm. (2.5 pounds, pullet) respectively. The pullet later reached a weight of 1,370 gm. (3 pounds) at an age of 298 days. The cockerel crowed vociferously and the pullet laid two eggs while under observation. Most of the other chickens of this lot, however, developed the characteristic inability to use their legs. Nevertheless many of them continued to gain weight in spite of the fact that they were almost entirely helpless. It is possible that these individuals did not acquire the habit of eating enough of the paper fiber and consequently their digestions became impaired with the subsequent development of "weak legs." On diets where no corn or other material furnishing plant pigments of the xanthophyll or carotin type was present the absence of pigmentation in the epidermal scales on the legs and the ear lobes was noticeable, testifying to the well known fact that the coloring matter in these parts comes from the food. Their combs and wattles were in most cases anemic.

The chicks thus far considered had presumably been reared under outdoor conditions and were 3 to 5 weeks old before they were selected for experiment. Drummond has expressed the conviction that, "The age at which the chick is brought under the influence of these artificial conditions is apparently the chief determining factor in its subsequent development, other conditions being the same." We therefore hatched a large number of White

Leghorn chicks in the laboratory. These were fed in groups, in a diversity of ways, our aim being to supply suitable protein, salts, and "vitamines" of both the water-soluble and fat-soluble types. Green food was never used. "Roughage" or "ballast" was furnished in the form of moist paper pulp. Later, many of the chicks ate in addition considerable quantities of the blotting paper with which the bottoms of the cages were covered. The fat-soluble "vitamine" which probably is ordinarily supplied by green food was furnished by a liberal amount of butter fat incorporated in the various rations fed.

It was soon made evident in these newer trials that chicks directly from the incubator can reach several times their initial weight, though fed on the mixtures selected by us, quite as satisfactorily as when the experiments were begun with chicks 3 to 4 weeks old. The rations used contained all of the nutrients which our experience with rats had led us to assume might be essential for growing chicks and in fairly similar proportions. Such changes as were made chiefly involved the physical state of the food and were in accordance with the results of our observation of the habits of these birds.

We are not yet prepared to furnish an interpretation of the unlike growth made by the chicks in the various experiments just referred to. The character of the food mixture used was varied so frequently that we cannot yet discuss this feature in detail. It seems more than fortuitous that the feeding of liberal proportions of gliadin or wheat gluten has had a favorable influence, especially in view of the high esteem in which wheat is held by poultrymen.

In all our different series a considerable number of the chicks developed the familiar "weakness of the legs" that is variously attributed to lack of exercise and other factors incident to indoor conditions. By changes in the diet or by outdoor conditions we have as a rule been unable to cure completely birds thus affected, although some throve fairly well while remaining permanently anatomically defective. Nevertheless a few of the experimental chicks continued to grow under the supposedly adverse conditions of caging and diet, ultimately gaining a weight of more than 1,250 gm. The appearance of two such birds is shown in the photographs taken at the age of 164 days and 1,278 (♀) and 1,267 (♂)

gm. of body weight respectively. Chicken 44 ♀ laid eggs after 178 days of age. The male bird, No. 57, reached a stage of development at which it crowed like an adult.

Despite the small proportion of the experimental birds which grew as well as did those here pictured, the success already achieved in the absence of dietary factors hitherto assumed to be essential for the growth of chickens, and also under supposedly adverse conditions of housing, encourages us to believe that all of the essentials for the nutrition and adequate growth of chickens under laboratory conditions can be ascertained, and that these will be controllable in much the same way as has proved possible in the case of other animals. The question of "roughage," suitable salts, proteins, and food hormones needs to be approached from new angles in the case of species that have characteristics of digestion and metabolism and structural requirements somewhat different from those of most mammals.

EXPLANATION OF PLATES.

PLATE 4.

FIGS. 1 and 2. Photographs of Rhode Island Red Chickens 5 ♂ and 6 ♂ (shown at an earlier age in our paper)[3] at the age of 322 days. They weighed between 6 and 7 pounds each.

PLATE 5.

FIGS. 3 and 4. White Leghorn Chickens 11 ♂ and 12 ♀ raised in the laboratory without green food from the age of 3 weeks. The photographs show them at an age of 271 days when they weighed 3.3 and 2.5 pounds respectively.

PLATE 6.

FIGS. 5 and 6. White Leghorn Chickens 44 ♀ and 57 ♂ raised in the laboratory without green food from the time they were a day old. The photographs show them at the age of 164 days when they weighed 2.8 pounds, each.

FIG. 1.

FIG. 2.

(Osborne and Mendel: Chickens in confinement.)

FIG. 3.

FIG. 4.

(Osborne and Mendel: Chickens in confinement.)

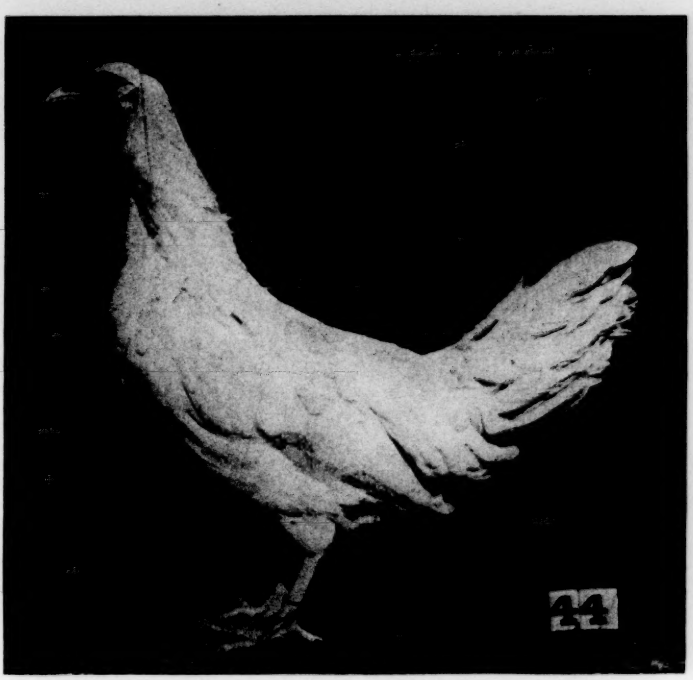

FIG. 5.

FIG. 6.

(Osborne and Mendel: Chickens in confinement.)

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

II. THE EFFECT OF SALTS ON THE PRECIPITATION OF ACID AND ALKALINE GELATIN BY ALCOHOL. ANTAGONISM.

By W. O. FENN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 14, 1918.)

In a previous paper[1] the writer described a method for studying the effects of electrolytes on gelatin which was found to possess many advantages. It consisted essentially in adding 95 per cent alcohol to 5 cc. of a gelatin-electrolyte mixture until an opaque precipitate was produced. The number of cc. of alcohol required to produce this precipitate was referred to as the "alcohol number" of gelatin. It can be quickly and accurately determined and is surprisingly sensitive to small variations in the electrolyte content.

The separate effects of acids, alkalies, and salts on the alcohol number of gelatin have already been described. The present paper deals with the effect of salts when combined with acids and alkalies. The effect of mixtures of two salts will form the subject of a later paper.

There is need in biology for a complete knowledge of the equilibrium between salts and acid proteins or alkaline proteins, especially in view of the antagonistic action of salts and acids in their effects on organisms, as described by Loeb,[2] by Osterhout[3] and by others. There is no evidence of antagonistic action between salts and alkalies in biological work, so far as the writer is aware. Instances of antagonism between salts and both alkalies and acids are, however, numerous in investigations on the physical chemistry of protein solutions.

[1] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279.

[2] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303. Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

[3] Osterhout, W. J. V., *J. Biol. Chem.*, 1914, xix, 517.

Thus Pauli[4] found that anions were most effective in counteracting the effects of acids on the coagulation temperature of egg white. Pauli and Handovsky[5] reported that anions were about equally effective in decreasing the viscosity of alkaline albumin, while the bivalent cations were more effective than the monovalent cations. Pauli[6] concluded that acids and alkalies ionize proteins and that neutral salts prevented this ionization. Hopkins and Savory[7] investigated the action of salts on the Bence-Jones protein from pathological urine. The protein coagulates at 55°C. but dissolves again at 100°C. in the presence of salts. The following relations obtained for this effect of the salts: $CaCl_2 > Na_2SO_4$ in acid solution, $Na_2SO_4 > CaCl_2$ in alkaline solution, and $Na_2SO_4 = CaCl_2$ in neutral solution. Chick and Martin[8] added neutral salts to acid and alkaline gelatin and found a lowering of the H and OH concentration respectively by measurements with a concentration cell. In acid albumin the polyvalent anions were most effective in this respect; in alkaline albumin, the polyvalent cations. Fischer[9] measured the swelling of gelatin in the presence of acid-salt mixtures and found that the effectiveness of the anions in hindering swelling by HCl increased in the order of the lyotropic series, while the effectiveness of the cations increased with the valence. NaCl was found to antagonize both acids and alkalies. The most important step in reaching an understanding of the antagonism between salts and acids was made by Procter[10] who investigated the HCl-NaCl-gelatin equilibrium in order to throw light on the strong dehydrating action of NaCl when used by tanners on the acid tissues of hides.

In the baking industry it is commonly understood that a flour with a high percentage of ash gives a stronger gluten in baking than one with a low percentage. This is attributed to the action of the salts of the flour in antagonizing the weakening effect on the gluten of the acids of fermentation. Hard water is also known to give a stronger gluten for the same reason. Salt is often added to overfermented dough to "neutralize" its acid. This fact has been established by Wood and Hardy.[11]

Other instances of antagonism between acids and salts will be given in a later paper on the precipitation of proteins in mixtures of electrolytes.

From the writer's experiments the following conclusions can be drawn concerning the antagonistic effect of salts on acids and alkalies in gelatin.

[4] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1907, x, 53.

[5] Pauli, W., and Handovsky, H., *Biochem. Z.*, 1910, xxiv, 239.

[6] Pauli, *Z. Chem. u. Ind. Kolloide*, 1910, vii, 241.

[7] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

[8] Chick, H., and Martin, C. J., *Kolloidchem. Beihefte*, 1913, v, 49.

[9] Fischer, M. H., *Kolloid-Z.*, 1915, xvii, 1.

[10] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *ibid.*, 1916, cix, 307.

[11] Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 38.

1. Salts with monovalent ions (like NaCl) decrease the effect of both acids and alkalies in gelatin.

2. Salts with bivalent (like $CaCl_2$) or trivalent cations decrease the effect of alkalies on gelatin but increase the effect of acid, except in high concentrations of salt or acid, where the effect is decreased.

3. Salts like Na_2SO_4 with bivalent or trivalent anions decrease the effect of acids on gelatin but increase the effect of alkalies, except in high concentrations, when the greater the con-

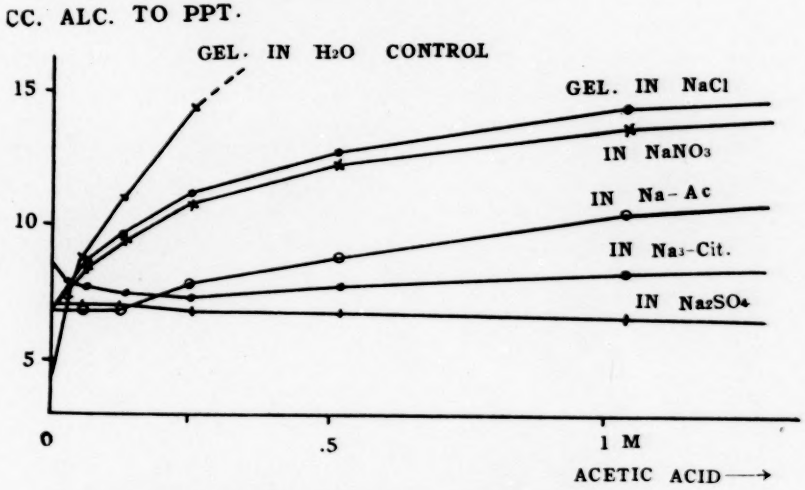

FIG. 1. Curves showing the effect on the alcohol number (ordinates) of increasing concentrations of acetic acid (abscissæ) in gelatin dissolved in water (control) and various sodium salts, all 0.125 M. The sodium salts antagonize the effect of the acetic acid and make precipitation by alcohol possible even in strong acetic acid. (See Table I.)

centration of either salt or alkali, the less the alcohol necessary for precipitation.

The effect of various sodium salts on gelatin plus acetic acid is illustrated in Fig. 1 and Table I. Acetic acid alone entirely prevents the precipitation of gelatin by alcohol except in comparatively low concentrations. In the presence of sodium salts, 0.125 M, however, precipitation by alcohol is possible even in

high concentrations of acetic acid. NaCl, for example, has, therefore, decreased the effect of acetic acid on the alcohol number of gelatin. But acetic acid has increased the effect of NaCl. Is this, then, a case of antagonism?

Osterhout has defined two electrolytes as *antagonistic when their combined effect is less than the additive effect*, the additive effect being the effect of the combination if each salt acts inde-

TABLE I.

Antagonism between Acetic Acid and Sodium Salts in Their Action on the Precipitation of Gelatin by Alcohol.

Concentration of acid.	Cc. alcohol to precipitate in gelatin plus					
	H_2O	NaCl	$NaNO_3$	Na acetate.	Na_2SO_4	Na citrate.
M						
4.17	No. ppt.	16.2	14.35	13.1	4.7	9.3
2.08	" "	15.4	15.4	12.0	6.1	9.0
1.04	" "	14.4	13.9	10.4	6.6	8.2
0.52	" "	12.8	12.4	9.0	6.8	7.7
0.26	14.35	11.2	10.9	7.9	6.9	7.4
0.13	10.95	9.75	9.55	7.1	7.0	7.5
0.065	8.85	8.8	8.6	7.0	7.1	7.8
0.032	7.3	7.9	7.8	6.8	7.1	8.0
0.016	6.15					
0.008	5.15					
0.004	4.5					
0.0	4.25	6.85	6.75	7.0	7.1	8.7

The first column shows the concentration of the acetic acid in the gelatin before adding alcohol. The succeeding columns show the number of cc. of 95 per cent alcohol which were necessary to produce a precipitate in 5 cc. of the acetic acid solution plus H_2O-gelatin, NaCl-gelatin, etc. In all cases the salt concentration was kept constant at 0.125 M, while the concentration of acetic acid varied. 3 per cent gelatin B was used throughout, and all the tubes stood over night at 26°C. before adding alcohol. The results of two determinations were averaged for each point. (See Fig. 1.)

pendent of the presence of the other.[12] This is of course the ideal criterion for antagonism. In the case of gelatin, however, it is frequently not easy to apply because the effects of the vari-

[12] For criteria of antagonism in biological work and the determination of the additive effect see Osterhout, *Bot. Gaz.*, 1914, lviii, 178; 1915, lx, 228.

ous electrolytes when each is used alone are quite different and their dilution curves are so dissimilar that the additive effects are difficult to determine. When the additive effect is not known, it is safe to say that *antagonism exists if the observed effect is less than that produced by the most strongly acting electrolyte* in the absence of the others (and in the same concentration in which it exists in the combination). When the observed effect is greater than this, there may still be some antagonism but it is very slight. This criterion will, therefore, be used throughout this paper in determining whether or not two electrolytes are antagonistic in their effects.

Returning to Fig. 1, the conclusion may be drawn that acetic acid is antagonized by sodium salts, the bivalent SO_4 ion and the trivalent citrate ion being most effective, and the acetate, which, like the citrate, yields an alkaline solution by hydrolysis, being more effective than the nitrate or chloride.

This experiment (Fig. 1) was designed to elucidate by means of gelatin an experiment of Loeb,[13] where 0.125 M sodium salts was found to prevent the penetration of acetic acid into the egg of *Fundulus*. The order of effectiveness found by Loeb was SO_4 > acetate, Cl > NO_3.

The relations of Na_2SO_4 to acid and alkaline gelatin are illustrated in Fig. 2. In this experiment the effect of increasing concentrations of Na_2SO_4 on gelatin dissolved in HCl, NaOH, and water was determined by the alcohol method. The effect of Na_2SO_4 on gelatin plus HCl is found to pass through a minimum. This minimum is probably an isoelectric point due to the neutralization of the effect of the H ions by the SO_4 anions. The proportions of SO_4 to H at this point are as 100: 30. Antagonism is shown by the fact that the combined effect of Na_2SO_4 and HCl at the minimum is less than that of the HCl alone. *Na_2SO_4 and HCl are, therefore, antagonistic in their effects on gelatin*, at least in certain concentrations.

The effect of Na_2SO_4 on gelatin plus NaOH is seen (Fig. 2 and Table II) to pass through a maximum and subsequently fall off. The effect of Na_2SO_4 alone is shown in the dotted line. At no point is the combined effect of Na_2SO_4 and NaOH less than the effect of either alone. *Therefore in low concentrations at least*

[13] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

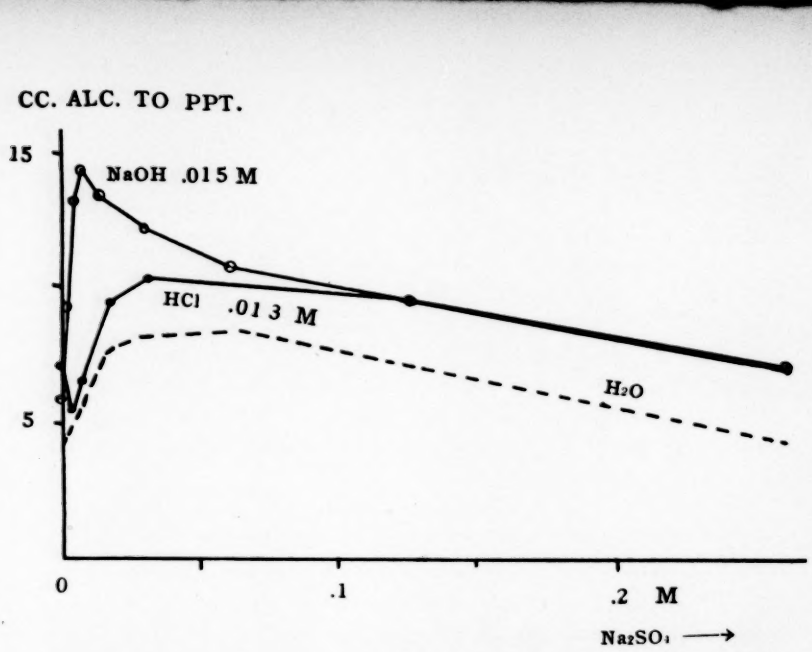

FIG. 2. Curves showing the effect of increasing concentrations of Na_2SO_4 (abscissæ) on the alcohol number of gelatin plus NaOH 0.015 M, HCl 0.013 M, and water (dotted line). The proportions of SO_4 to H at the minimum of the HCl curve are 100: 30. Na_2SO_4 is antagonistic to HCl but not antagonistic to NaOH in small concentrations. (See Table II.)

TABLE II.

Effect of Na_2SO_4 on the Alcohol Number of Gelatin plus HCl and NaOH.

Concentration of Na_2SO_4.	Cc. alcohol to precipitate in gelatin plus		
	H_2O	HCl, 0.013 M	NaOH, 0.015 M
M			
0.5	3.1	4.9	4.5
0.25	4.6	7.25	7.1
0.125	7.1	9.6	9.6
0.0625	8.3	10.2	10.8
0.0312	8.2	10.4	12.2
0.0156	7.7	9.3	13.3
0.0078	5.8	6.5	14.5
0.0039	5.1	5.5	13.2
0.00195	4.3	5.9	9.2
0.00097	4.1	6.5	7.75
0.00048	3.95	6.9	7.1
0.0	3.9	7.2	6.4

3 per cent gelatin C which stood over night at 26°C. before adding alcohol. Gelatin C is not so pure as gelatin B. Other details as in Table I. (See Fig. 2.)

NaOH and Na_2SO_4 are not antagonistic. Their relations in higher concentrations are like those for Na_3 citrate and NaOH in Fig. 3.

To determine the effects of higher concentrations of Na_3 citrate and NaOH on gelatin, the effect of increasing concentrations of Na_3 citrate was determined for gelatin made up in water, and in six concentrations of NaOH. The results for four of these concentrations and for water are plotted in Fig. 3 (see Table III). The concentrations of Na_3 citrate used are plotted on the abscissæ and the concentrations of NaOH simultaneously present in the gelatin are inserted on the curves. In the concentrations of NaOH chosen, no precipitate could be produced with alcohol unless Na_3 citrate was also present in sufficient amount. The more Na_3 citrate there is present the easier it is to precipitate. This shows antagonism. At points A and B, moreover, the combined effect is less than the effect of either Na_3 citrate or NaOH alone in those concentrations. An examination of the ordinates at B shows that small amounts of NaOH increase the alcohol number of gelatin plus Na_3 citrate, but larger amounts decrease it. This is in agreement with the fact that small amounts of NaOH hinder the precipitation of gelatin by Na_2SO_4 and Na_3 citrate, while larger amounts favor it.[14] Conversely, it may be inferred from this figure, taken in conjunction with the experiments in Fig. 2, that small amounts of Na_2SO_4 or Na_3 citrate increase the alcohol number of gelatin plus NaOH, while higher concentrations decrease it.

From this experiment the conclusion may therefore be drawn that *Na_2SO_4 and NaOH are not antagonistic in weak concentrations (of both) but that they are antagonistic in higher concentrations.*

In Fig. 4 and Table IV are shown the effects of increasing concentrations of $CaCl_2$ on the alcohol number of gelatin dissolved in water, NaOH, and acetic acid. In NaOH, the curve passes through a sharp minimum. This is due in part at least, to the formation of $Ca(OH)_2$, a small amount of which precipitated. It is probable that there is also some residual antagonistic effect upon the gelatin. In their relations to alkaline gelatin $MgCl_2$ and $AlCl_3$ behave like $CaCl_2$, the curves passing through similar minima. In acid gelatin, however, $MgCl_2$ behaves more like

[14] Figures for this will appear in a subsequent paper on the precipitation of gelatin by mixtures of electrolytes.

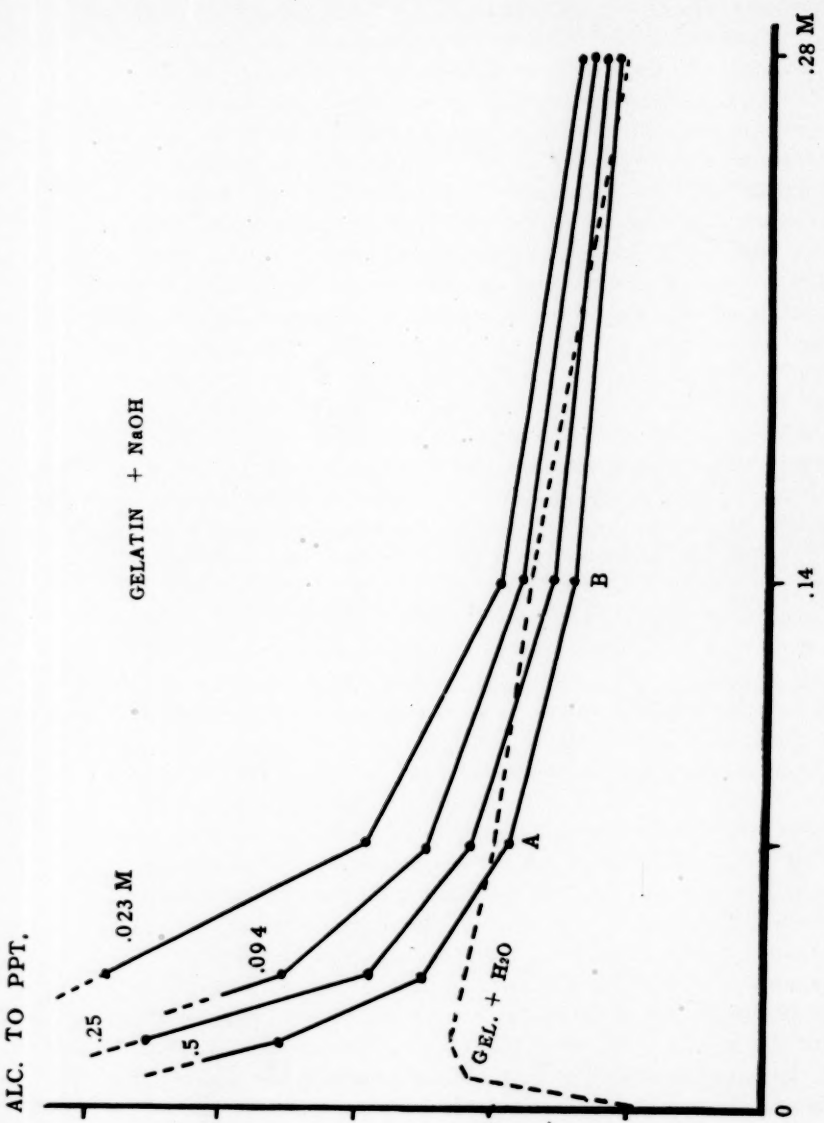

FIG. 3. Curves showing the effect of Na citrate on the alcohol number of gelatin plus NaOH of various concentrations, as noted on the curves. In pure NaOH no precipitation by alcohol is possible. (See Table III.)

NaCl because it fails to give the sharp maximum which $CaCl_2$ gives in its effect on acetic acid-gelatin, as shown in Fig. 4.

A sharp maximum like that shown for $CaCl_2$ plus acetic acid has been shown to be characteristic of the effects of acid alone on the alcohol number of gelatin. It is not found in the pure $CaCl_2$ curve as shown by the dotted line. Furthermore, an examination of these curves reveals the fact that at no point is the combined effect of $CaCl_2$ and acetic acid less than the effect of either $CaCl_2$ or acetic acid alone. $CaCl_2$ and acetic acid, at least in small concentrations, are not antagonistic in their effects.

TABLE III.

Effect of Sodium Citrate on the Alcohol Number of Gelatin plus NaOH.

Concentration of Na citrate.	Cc. alcohol to precipitate in gelatin plus NaOH.						
	0.0 M	0.0235 M	0.047 M	0.094 M	0.127 M	0.254 M	0.508 M
M							
0.28	5.4	7.0	6.5	6.5	6.6	6.1	5.6
0.14	8.8	9.7	9.6	8.9	8.9	7.9	7.1
0.07	10.0	14.7	13.7	12.4	13.6	10.7	9.4
0.035	10.9	24.4	22.0	17.8	16.5	14.6	12.6
0.0175	11.5	No ppt.	30.0+	No ppt.	22.7	22.8	17.8
0.0087	10.7	" "	No ppt.	" "	32.0+	30.0	30.0
0.0043		" "	" "	" "	No ppt.	No ppt.	No ppt.
0.0	4.5	" "	" "	" "	" "	" "	" "

2 per cent gelatin A for 15 to 20 hours at 30°C. before adding alcohol. Other details as in Table I. (See Fig. 3.)

In higher concentrations of $CaCl_2$, however, it is antagonistic to acids. Thus Fig. 5 and Table V show that acetic acid completely prevents the precipitation of gelatin by alcohol in concentrations greater than 0.1 M (see dotted line in the H_2O curve) but that when $CaCl_2$ 1.5 M is also present, precipitation is possible even in high concentrations of acetic acid. A comparison of this result with the acetic acid curves in gelatin plus sodium salts (Fig. 1) shows that although $CaCl_2$ does antagonize acids it is very much less effective in this respect than are the sodium salts, particularly those with polyvalent anions.

In low concentrations, therefore, *$CaCl_2$ is not antagonistic to acids but in higher concentrations it is.*

This experiment is of particular interest from the biological point of view, since Loeb[15] has found that the effectiveness of cations in hindering the penetration of acetic acid into the egg of *Fundulus* increases with the valence, and this result is contrary to the suggestion that antagonism is due to oppositely charged ions. While this result cannot be paralleled in gelatin by the alcohol method, the experiment recorded in Fig. 5 shows without a doubt that antagonism between acids and salts with biva-

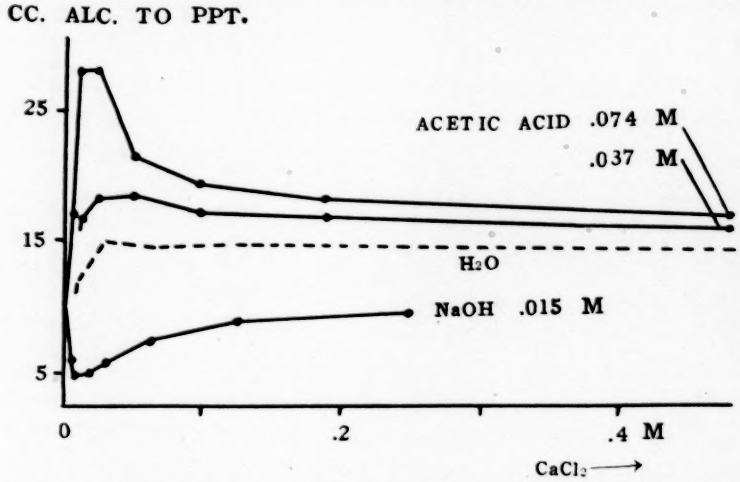

FIG. 4. Curves showing the effect of $CaCl_2$ on the alcohol number of gelatin plus NaOH and acetic acid. $CaCl_2$ is antagonistic to NaOH but not antagonistic to acetic acid. (See Table IV.)

lent cations is possible in gelatin. The writer has also found that $CaCl_2$ 1.5 M will antagonize HNO_3 and $AlCl_3$ in the same way.

Fischer[16] has found, in studying the swelling of gelatin in acids, that the effectiveness of cations in hindering this swelling increases with the valence of the cation. Thus he found that $FeCl_3$ hinders swelling in HCl more than $CaCl_2$. This is in accord with Loeb's results but is the reverse of the evidence so far obtainable by the alcohol method, where no indication of antagonism between HCl and $AlCl_3$ has as yet been found. As it is impossible to follow the effects of HCl and $AlCl_3$ by this method,

[15] Loeb, *J. Biol. Chem.*, 1916, xxvii, 365.

[16] Fischer, Œdema and Nephritis, New York, 2nd edition, 1915.

TABLE IV.

Effect of $CaCl_2$ on the Precipitation by Alcohol of Acid and Alkaline Gelatin.

In acetic acid.*			In NaOH.†	
Concentration of $CaCl_2$.	Cc. alcohol to precipitate in gelatin plus		Concentration of $CaCl_2$.	Cc. alcohol to precipitate in gelatin plus NaOH, 0.015 M
	Acetic acid 0.0371 M	Acetic acid 0.0743 M		
M			M	
1.5	15.6	16.4	0.25	9.9
0.75	16.0	17.0	0.125	9.1
0.375	16.7	17.8	0.0625	7.8
0.188	17.2	18.5	0.0312	6.2
0.094	17.5	19.5	0.0156	5.2
0.047	18.8	21.5	0.0078	5.1
0.024	18.6	27.7	0.0039	6.3
0.012	16.9	27.7	0.00195	7.6
0.006	13.8	17.1	0.00097	8.4
0.003	11.4	14.3	0.00048	9.1
0.0	8.65	10.6	0.0	11.0

* 2 per cent gelatin A for 24 hours before adding alcohol.
† 3 per cent gelatin B for 15 hours at 26°C.

The NaOH and the acetic acid curves are not strictly comparable but as they are so different there is no confusion in plotting them together. Gelatin B was not so pure a grade of commercial gelatin as gelatin A. Data for the effect of $CaCl_2$ on pure gelatin are given in Table II of the first paper of this series and are plotted with the data of this table in Fig. 4. Other details as in Table I.

except in very low concentrations, it is possible that some antagonism exists. $AlCl_3$ is certainly less effective in this respect, however, than $CaCl_2$.

In this connection, reference should be made to the conclusions of Chick and Martin[17] concerning the effects of acids and alkalies on salts in albumin solutions. They found that small amounts of NaOH and HCl hindered the precipitation of albumin by Na_2SO_4 and $CaCl_2$ respectively, but larger amounts favored it.

[17] Chick and Martin, *Biochem. J.*, 1913, vii, 380.

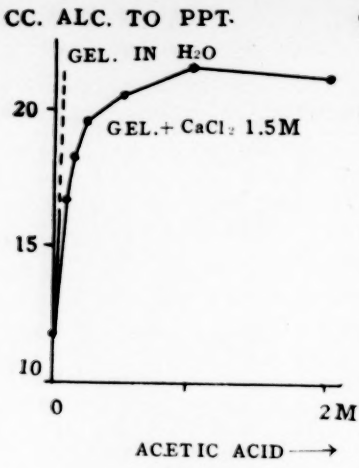

FIG. 5.

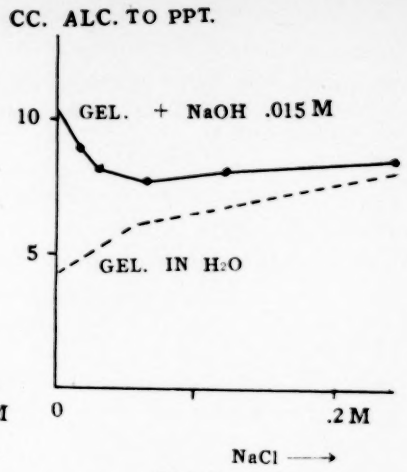

FIG. 6.

FIG. 5. Curves showing how strong concentrations of $CaCl_2$ (1.5 M) make precipitation of the gelatin by alcohol possible even in high concentrations of acetic acid. This shows antagonism. (See Table V.)

FIG. 6. Curves showing the effect of NaCl on H_2O-gelatin (dotted line) and NaOH-gelatin. Antagonism is shown by the drop of the NaOH curve. (See Table VI.)

TABLE V.

Antagonism between High Concentrations of $CaCl_2$ and Acetic Acid.

Acetic acid.*		Acetic acid in gelatin plus $CaCl_2$ 1.5 M†	
Concentration of acetic acid.	Cc. alcohol to precipitate.	Concentration of acetic acid.	Cc. alcohol to precipitate.
M		M	
0.13	No ppt.	4.17	19.3
0.065	11.6	2.08	21.4
0.033	9.25	1.04	21.7
0.016	7.75	0.52	20.7
0.008	6.85	0.26	19.8
0.0	4.95	0.13	18.2
		0.065	16.7
		0.0325	15.7
		0.0162	15.1
		0.008	14.1
		0.004	13.7
		0.0	13.2

*2 per cent gelatin A over night at 31°C.

†2 per cent gelatin A. Temperature regulator broke and the temperature varied; therefore it was not strictly comparable. Other details as in Table I. (See Fig. 5.)

The effect of NaCl on gelatin dissolved in NaOH is shown in Fig. 6 and Table VI. The effect of NaCl on pure gelatin is a gradual increase in the alcohol number, as shown by the dotted line. Its effect on gelatin made up in NaOH, 0.015 M, is to decrease the alcohol number until a minimum is reached, after

TABLE VI.

Antagonism between NaCl and NaOH in Their Effects on Gelatin.

Concentration of NaCl.	Cc. alcohol to precipitate in gelatin plus	
	H_2O	NaOH, 0.015 M
M		
0.25	8.0	8.45
0.125	6.85	8.05
0.0625	6.15	7.8
0.0312		8.1
0.0156		9.0
0.0	4.4	10.4

3 per cent gelatin B. Stood over night at 26°C. before adding alcohol. Other details as in Table I. (See Fig. 6.)

which the alcohol number increases gradually as in the pure NaCl curve. This shows antagonism because the combined effect of NaOH and NaCl is less than the effect of one of the electrolytes (NaOH) alone.

SUMMARY.

1. Monovalent salts (like NaCl) are antagonistic to both acids and alkalies in all concentrations.

2. Salts with bivalent cations (like $CaCl_2$) are antagonistic to alkalies but not antagonistic to acids (except in high concentrations).

3. Salts with bivalent anions (like Na_2SO_4) are antagonistic to acids but not antagonistic to alkalies except in high concentrations when the greater the concentration of either alkali or salt, the less the alcohol necessary for precipitation.

THE EFFECT OF POTASSIUM BROMATE UPON ENZYME ACTION.

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(Received for publication, November 14, 1917.)

One of the most recent important advances in the practical art of breadmaking has been the introduction into the dough of a mixture of mineral nutrients known as Arkady Yeast Food.[1] This material, which was devised in the laboratories of the Mellon Institute of Pittsburg, includes salts of calcium and ammonium and a minute amount (1 part in 200,000) of potassium bromate.

The functions of calcium and ammonium as yeast foods are fairly well understood; but the reasons for the favorable action of the bromate remained somewhat obscure. It seemed possible that this salt might exert a specific stimulating action upon the proteolytic enzymes active in the fermentation. It had been shown by Chittenden and his associates[2] that bromides in certain dilutions had a marked accelerating effect on the action of ptyalin, pepsin, and pancreatin. Wohlgemuth[3] noted the same effect on ptyalin and pancreatin. More recently Thomas[4] reports a strongly stimulating influence of bromides upon amylase; and Robertson[5] in an exhaustive study of the action of trypsin on casein notes a stimulating action of potassium bromide. On the other hand, Senter,[6] and Battelli and Stern,[7] and Jappelli[8] found

[1] Allen, R. M., *Baker's Weekly*, 1916, xviii, 31.

[2] Chittenden, R. H., and Painter, H. M., *Tr. Conn. Acad. Arts and Sc.*, 1885–88, vii, 60. Chittenden, R. H., and Allen, S. E., *ibid.*, 84. Chittenden, R. H., and Cummins, G. W., *ibid.*, 108.

[3] Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 10.

[4] Thomas, A. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1501.

[5] Robertson, T. B., *J. Biol. Chem.*, 1906–07, ii, 317.

[6] Senter, G., *Z. physikal. Chem.*, 1903, xliv, 257.

[7] Battelli, F., and Stern, L., *Arch. internat. Physiol.*, 1907, iv, 465.

[8] Jappelli, A., *Atti XXII cong. med. int. Roma*, 1913, 405.

that oxidizing enzymes were inhibited by bromides. All these investigations were made with rather strong concentrations of salts; and nothing, so far as we are aware, has been published on the question of the action of bromates upon the enzymes, trypsin and pancreatin. An investigation of this point was therefore undertaken at the request of the Ward Baking Company of New York.

Effects on Trypsin.

The technique of the trypsin experiments was as follows: A buffer solution was prepared, made up of 40 cc. of 0.067 M K_2HPO_4, 10 cc. of 0.067 M KH_2PO_4, and 50 cc. of water; pH value 7.1. Definite amounts of casein and trypsin were added as stated in the protocol of the individual experiments, and to a series of bottles of the resulting solution were added varying amounts of potassium bromate. The bottles were then incubated at 37°C. for the periods stated in the protocols.

At the end of the incubation period the amino-acid content of each bottle was determined by the following method, and the excess produced by the action of the trypsin found by subtracting the amino-acid content of a control containing casein alone. Each bromate dilution was tested in triplicate.

Method of Titration.

1. Add phenolphthalein to incubated solution.
2. Add $Ba(OH)_2$ (saturated solution) to deep red color—removal of phosphates.
3. Pour into graduated cylinder.
4. Rinse incubation bottle with small quantity of distilled H_2O. Pour rinsings into cylinder.
5. Make up to 100 cc. with distilled H_2O.
6. Filter through paper into bottle.
7. Measure off 50 cc. of filtrate and pour into cylinder.
8. Neutralize to faint pink with 0.2 N HCl.
9. Add 0.2 N NaOH to desired titration color.
10. Add 15 cc. of neutralized HCHO.
11. Titrate with 0.2 N NaOH to same color as in (9).
12. Express results in terms of cc. of 0.2 N NaOH to neutralize after addition of neutralized HCHO.

In a series experiment, preserve control by addition of $CHCl_3$ and keeping in a dark place.

Experiment I.—June 11, 1917. 2 gm. of casein, 0.4 gm. of Merck's trypsin, per 100 cc. of solution. Digestion period, 4 hours.

Solution.	Initial pH.	Final pH.	Titrations of 50 cc. portions.*				
			Initial amino-acids. Cc. 0.2 N NaOH to neutralize.	Final amino-acids. Cc. 0.2 N NaOH to neutralize.		Increase in amino-acids.	Titratable acid. Final cc. 0.2 N NaOH.	
Buffer + casein.....	6.1	5.9	1.80	1.85		0.05	7.5	
				7.15			11.60	
" " + trypsin.....	6.1	5.9	1.80	6.75	6.65	4.85	11.45	11.70
				6.05			12.00	
				6.85			11.80	
" " " + $KBrO_3$ 1 : 10,000.....	6.1	5.9	1.80	6.45	6.55	4.70	12.30	12.10
				6.30			12.15	
				7.00			12.45	
" " " " 1 : 50,000.....	6.1	5.9	1.80	6.65	6.95	5.15	12.00	12.15
				7.20			11.95	
				8.60			10.70	
" " " " 1 : 100,000.....	6.1	5.9	1.80	6.95	7.90	6.10	11.05	10.90
				8.20			10.90	

* Results expressed to nearest 0.05.

The results of check determinations in this experiment were a little irregular, but the tubes containing 1 part of bromate in 100,000 showed a significant increase in digestion.

Experiment II.—June 16, 1917. 2 gm. of casein, 0.4 gm. of Merck's trypsin, per 100 cc. of solution. Digestion period, 4½ hours.

Solution.	Titration of 50 cc. Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralize.
Buffer + casein + trypsin.	5.2 5.9 5.7 6.0
" " " + $KBrO_3$ 1: 10,000	6.2 6.4 6.1 5.8
" " " " 1: 50,000	5.7 5.9 5.9 6.0
" " " " 1: 100,000	6.6 6.2 6.3 6.0
" " " " 1: 200,000	6.4 6.7 6.8 7.2
" " " " 1: 300,000	6.2 6.2 6.1 5.8
" " " " 1: 500,000	5.9 6.0 5.6 5.0

The results of this experiment again show considerable individual variations, but with higher amino-acid contents at bromate concentrations of 1 in 100,000 and 1 in 200,000, the latter being most marked.

Experiment III.—June 29, 1917. 2 gm. of Merck's casein, 0.1 gm. of Fairchild Brothers and Foster's trypsin, per 100 cc. Digestion period, 3½ hours.

Solution.	Titration of 50 cc. Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralize.
Buffer + casein + trypsin.	2.4 2.0 2.1 2.0
" " " + $KBrO_3$ 1: 10,000	2.7 2.7 2.7 2.7
" " " " 1: 50,000	3.3 3.3 3.3 3.3
" " " " 1: 100,000	3.6 3.1 3.4 3.6
" " " " 1: 150,000	4.0 5.3 4.5 4.1
" " " " 1: 200,000	2.6 4.2 3.4 3.5
" " " " 1: 500,000	3.0 2.7 2.7 2.5

In this experiment the digestion increases regularly with decreasing amounts of bromate to a concentration of 1 part in 150,000 and then decreases again with further dilution of the salt. Here as in other experiments it appears, however, that even certain high concentrations of bromate (1 part in 10,000) are rather favorable than otherwise to the process of enzyme action.

Experiment IV.—July 13, 1917. 2 gm. of Merck's casein, 0.2 gm. of Fairchild Brothers and Foster's trypsin, per 100 cc. Digestion period, 3 hours.

Solution.					Titration of 50 cc.	
					Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein + trypsin.					2.7 2.85 2.7	2.75
"	"	"	+ $KBrO_3$	1:100,000	2.8 3.0 3.1	3.0
"	"	"	"	1:120,000	3.25 3.1 4.4	3.6
"	"	"	"	1:140,000	3.85 3.3 3.6	3.6
"	"	"	"	1:160,000	4.1 4.25 4.1	4.15
"	"	"	"	1:180,000	4.25 4.0 4.2	4.15
"	"	"	"	1:200,000	5.5 5.8 5.8	5.7
"	"	"	"	1:250,000	4.9 4.7 4.0	4.5

Again the result is an increase of enzyme action under the influence of bromate up to a concentration of 1 part in 200,000. In both these last experiments the digestive action was more than doubled by the stimulating effect of the bromate.

Effects on Pancreatin.

The influence of bromate upon the action of pancreatin was next studied in the same way. The buffer solution in this case was made up with 50 cc. of 0.067 M K_2HPO_4, 17.5 cc. of 0.2 N NaOH, and 32.5 cc. of water, giving an initial pH value of 10.8. The titration method was the same as outlined above.

Experiment V.—July 30, 1917. 3 gm. of Merck's casein, 0.5 gm. of Merck's pancreatin, to 100 cc. Digestion period, 5½ hours.

Solution.	Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.		
" "		0.85
" "		
" " + pancreatin.	4.00	
" " "	4.20	3.95
" " "	3.70	
" " " + $KBrO_3$ 1:100	3.90	
" " " "	3.45	3.95
" " " "	4.45	
" " " " 1:500	3.85	
" " " "	3.60	3.60
" " " "	3.40	
" " " " 1:1,000	2.70	
" " " "	3.10	3.00
" " " "	3.10	
" " " " 1:10,000	3.70	
" " " "	3.35	3.50
" " " "	3.50	

This experiment appears to indicate that bromate in strong concentrations (1:100 to 1:10,000) exerts an inhibitive action upon pancreatin.

Experiment VI.—July 30, 1917. 3 gm. of Merck's casein, 0.5 gm. Merck's pancreatin, to 100 cc. Digestion period 4 hours.

Solution.	Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.	0.80	
" "		
" "		
" " + pancreatin.	2.95	
" " "	2.75	2.90
" " "	2.95	
" " " + $KBrO_3$ 1:200	2.85	
" " " "	2.85	2.80
" " " "	2.80	
" " " " 1:400	2.40	
" " " "	2.60	2.50
" " " "	2.55	
" " " " 1:600	2.10	
" " " "	2.35	2.25
" " " "	2.30	
" " " " 1:50,000	2.60	
" " " "	2.35	2.45
" " " "	2.40	
" " " " 1:100,000	2.50	
" " " "	2.65	2.60
" " " "	2.60	
" " " " 1:150,000	3.00	
" " " "	3.15	2.95
" " " "	2.75	
" " " " 1:200,000	3.45	
" " " "	3.50	3.70
" " " "	4.10	
" " " " 1:250,000	3.30	
" " " "	3.75	3.55
" " " "	3.55	

Again the stronger bromate solutions (1:200 to 1:100,000) appear to inhibit the action of the enzyme, while higher dilutions (1:200,000 and 1:250,000) show a distinct increase in digestion products.

Experiment VII.—Aug. 10, 1917. 2 gm. of Merck's casein, 0.5 gm. of Merck's pancreatin to 100 cc. In this experiment large portions of the solution (95 cc.) were put up in bottles and incubated for 24 hours, bacterial action being held in check by 5 cc. of toluene on the surface of the liquid in each bottle. The bottles were shaken every 10 minutes for the first 4 hours.

Solution.					Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.					0.90	
"	"				0.90	0.90
"	"				0.90	
"	"	+ pancreatin.			4.75	
"	"	"			4.70	4.70
"	"	"			4.70	
"	"	"	+ $KBrO_3$	1:100	4.60	
"	"	"	"		4.50	4.50
"	"	"	"		4.40	
"	"	"	"	1:500	4.30	
"	"	"	"		*4.45	4.35
"	"	"	"		4.35	
"	"	"	"	1:1,000	4.20	
"	"	"	"		4.45	4.35
"	"	"	"		4.35	
"	"	"	"	1:10,000	4.70	
"	"	"			4.60	4.70
"	"	"			4.85	
"	"	"	"	1:50,000	4.90	
"	"	"	"		4.90	4.90
"	"	"	"		4.85	
"	"	"	"	1:100,000	5.30	
"	"	"	"		5.35	5.20
"	"	"	"		4.90	
"	"	"	"	1:250,000	4.90	
"	"	"	"		5.25	5.10
"	"	"	"		5.10	
"	"	"	"	1:500,000	4.85	
"	"	"	"		4.85	4.85
"	"	"	"		4.90	
"	"	"	"	1:1,000,000	4.60	
"	"	"	"		4.50	4.60
"	"	"	"		4.75	

The same results are again apparent, slight inhibition by concentrations of 1: 100 to 1: 1,000, and slight stimulation by concentrations of 1: 50,000 to 1: 500,000.

CONCLUSIONS.

1. Potassium bromate appears to exert consistently favorable influence upon the digestion of casein by trypsin *in vitro* in the dilutions studied, the action being most marked at bromate concentrations of 1 part in 100,000 to 1 part in 200,000.

2. Potassium bromate in concentrations of 1 part or more in 10,000 appears to exert a slight inhibitive influence upon the digestion of casein by pancreatin, while in higher dilutions (1 part in 200,000 or 1 part in 250,000) it appears to exert a stimulating action.

CELL PENETRATION BY ACIDS.

IV. NOTE ON THE PENETRATION OF PHOSPHORIC ACID.

By W. J. CROZIER.

Contributions from the Bermuda Biological Station for Research, No. 83.

(Received for publication, December 12, 1917.)

I. Measurements of the relative speeds with which acids penetrate protoplasm, as carried out by different observers upon widely different tissues containing natural indicators, have shown in general a noteworthy agreement with regard to the way in which the various acids are arranged in order of their penetrating ability (Harvey, 1914; Crozier, 1916, *a*, *b*; Haas, 1916, *a*). This must indicate some fundamental fact about the organization of protoplasm, since some of the relations observed are such as might not be predicted. It is, then, of interest to examine an instance in which minor disagreement has been found. The agreement referred to obtains in acid solutions which are 0.01 N by titration, and it has been shown (Crozier, 1916, *a*, *b*) that, owing to the several forms of the penetration curves, the sequence of increasing penetrating ability may vary with the concentration at which the comparisons are made; and it was pointed out that a similar condition obtained with regard to comparisons at any one hydrogen ion concentration. Any special influence affecting the determination of the change of indicator by a particular acid may cause the form of its penetration curve to be different with different tissues, according to the variation of this special factor. Thus, among other possibilities, variations in the composition of the surface layer of the cell, or in the "buffer" conditions under which the intracellular titration takes place, or variations in the behavior of the different indicators used, might be appealed to to explain observed differences in the relative order of penetration for different tissues; and it might be expected that an acid whose salts have special significance in connection with protoplasm would be one to show characteristic variations from cell to cell.

Now, Haas (1916, *a*) found that for one kind of plant material used in his experiments, the petals of *Browallia*, phosphoric acid produced the color change signifying penetration more rapidly, at 0.01 N H^+ concentration, than did oxalic, tartaric, lactic, citric, or acetic acids; whereas, with the perianth cells of a hyacinth, this took place more slowly than in oxalic acid. Harvey (1914) observed the penetration of phosphoric acid, at this concentration, to occur with about the same speed as malonic or tartaric, but more slowly than with lactic or oxalic.

For the utilization of the results of these penetration studies it was necessary to know something about the action of this acid upon the surface of cells; so experiments were made to determine its behavior with the tissue of *Chromodoris zebra;* the results throw some light upon the factors which may cause the apparent speed of penetration by different acids, as observed by this method, to vary in different tissues.

II. The technique in these measurements of the penetration of *o*-phosphoric acid was essentially as has previously been explained (Crozier, 1916, *a*, *b*). Some observations were repeated for certain acids giving penetration curves lying near that of phosphoric; these experiments sufficed to show that the curves previously published could be relied upon for comparison. Two series of experiments were made with phosphoric acid, at the same time but at different temperatures. The average penetration times obtained in the two series, as shown in Table I, differ to

TABLE I.

The apparent time required for the penetration of mantle tissue of *Chromodoris* by H_3PO_4 at different concentrations and at two temperatures; penetration time in minutes; figures in brackets obtained only in favorable cases (about 75 per cent).

Temperature.	Concentration, normal.							
	1.34	0.67	0.134	0.067	0.013	0.010	0.007	0.005
°C.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*		
24.8	2.5	4.2	5.5	7.1	14.3	[20]	∝	∝
27.0	2.2	3.7	4.9	6.0	13.0	[18.0]	∝	∝

a degree which will serve to illustrate the delicacy of the method (Fig. 1). Observations were made at 27.0° and at 24.8°; the temperature of the sea water from which the chromodorids had been recently obtained was 24–25°.

III. The average apparent penetration times of phosphoric acid, from different concentrations, are plotted in Fig. 2. It is seen that from low concentrations the penetration appears abnormally slow, in comparison with some other acids; or that the increase in speed of penetration with higher concentrations is unusually rapid. From Fig. 2 it will be evident that at any particular concentration (*e.g.*, 0.01 N) the apparent relative position of phosphoric acid in the series will depend upon the intensity of

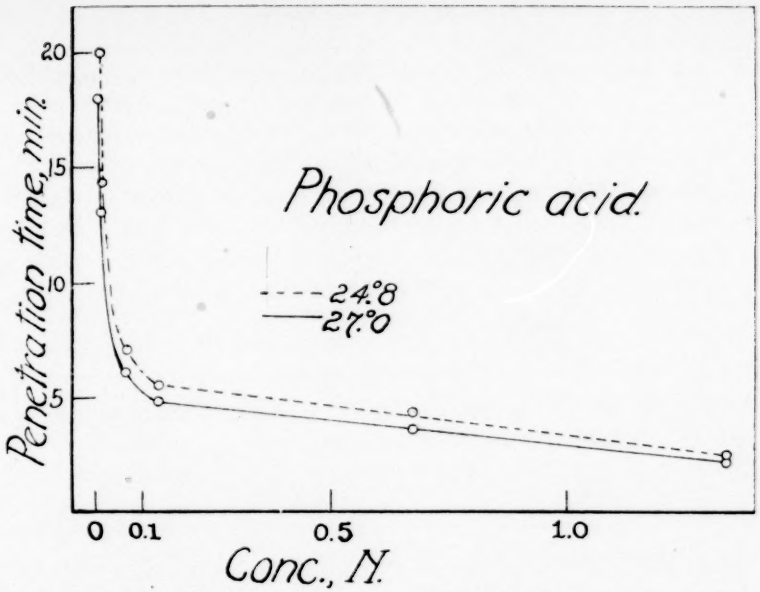

FIG. 1. The apparent penetration of phosphoric acid at two temperatures.

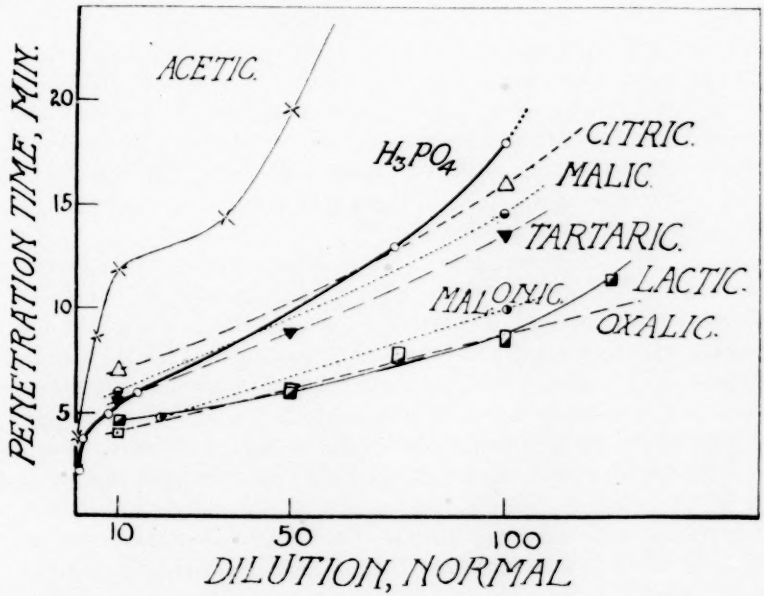

FIG. 2. The apparent penetration of H_3PO_4 compared with that of other acids (27.0°).

the factor responsible for the rapidly increasing slope of its penetration curve. This factor is probably concerned, in part, with differences in the kind and quantity of intracellular materials with which the phosphoric acid may react to produce "buffer" substances. Thus in four tissues which are available for comparison, the penetration relations of H_3PO_4 appear as follows (at 0.01 N).

TABLE II.

Source of tissue.	Transition point of indicator.	Order of apparent penetration.
	pH	
Chromodoris.	5.6	Oxalic, lactic > malonic > tartaric > malic > citric > H_3PO_4 > acetic.
*Stichopus.**	(3.0?)	Lactic, oxalic > malonic, tartaric, H_3PO_4 > malic, citric > acetic.
Browallia.†	6.0–5.0‡	H_3PO_4 > oxalic > tartaric > lactic, citric > acetic.
Hyacinth.†	4.0‡	Oxalic > H_3PO_4 > tartaric > lactic > citric, acetic.

* Harvey, 1914. † Haas, 1916, *a*. ‡ Haas, 1916, *b*.

These differences appear in further part accounted for by the greater density of the *Chromodoris* tissue. The case of H_3PO_4 is somewhat different from that of caprylic acid, whose penetration cannot be observed at all by this method because of its relatively low solubility in the aqueous phase containing the indicator, although saturated solutions of this acid will produce color change in water solutions of the pigment.

IV. The actual speed of protoplasmic penetration by H_3PO_4 is probably much greater than its apparent magnitude. This is shown by the way in which the speed of diffusion rapidly increases with increasing concentration. In solutions of 0.01 N with respect to H^+, Haas (1916, *a*) observed that this acid penetrated about as rapidly as other strong acids (HCl, HNO_3), but less rapidly than acetic, formic, or lactic acids. At 0.056 N total concentration, H_3PO_4 solutions are about 0.01 N in hydrogen ions. From the curve in Fig. 1 it is seen that at this concentration the penetration time of phosphoric acid is 6.4 minutes; under similar conditions the figures for several other acids are: HCl, 7.6; HNO_3, 8.4; formic < 1.0; lactic, < 2.0. The inward diffusion of H_3PO_4,

at higher concentrations, appears, then, to be somewhat more rapid than its ionization would demand.

The figures in Table I show that at dilutions above 0.01 N the penetration of phosphoric acid is not detectable with the tissue used. It requires an external concentration of 0.01 N acid to affect color transition. In favorable cases it is found that this color transition occurs in about 18 minutes. If this limiting concentration is subtracted from all higher concentrations studied, a truer idea is obtainable of the real penetrating ability of the acid. It is necessary also to subtract from the reciprocal of each observed penetration time the reciprocal of that for the limiting quantity of acid. This reduction is carried out in Table III. The same procedure has been used with data previously obtained for other acids, and leads to some interesting conclusions; it is believed to yield information concerning the primary action of acids upon the surfaces or outer layers of cells, and in the case of strong acids it can be shown that the peculiarities of their relations depend upon reactions with proteins.

TABLE III.

The corrected concentrations and speeds of penetration for H_3PO_4 (27°). Concentration, N; speed, $\frac{1}{\text{min.}} \times 10^3$ (27°).

Concentration.		$\frac{1{,}000}{P.\ T.'}$
Nominal.	Corrected.	
1.34	1.33	398.5
0.67	0.66	215.5
0.134	0.124	148.5
0.067	0.057	111.5
0.0134	0.0034	20.4
0.010	—	—
0.007	—	—

Fig. 3 exhibits the penetration relations of phosphoric acid as shown in the corrected data (Table III); measurements with HCl are included for comparison. The significant feature of these curves lies in the fact that they are composed of two straight lines, representing the respective predominance of two different proc-

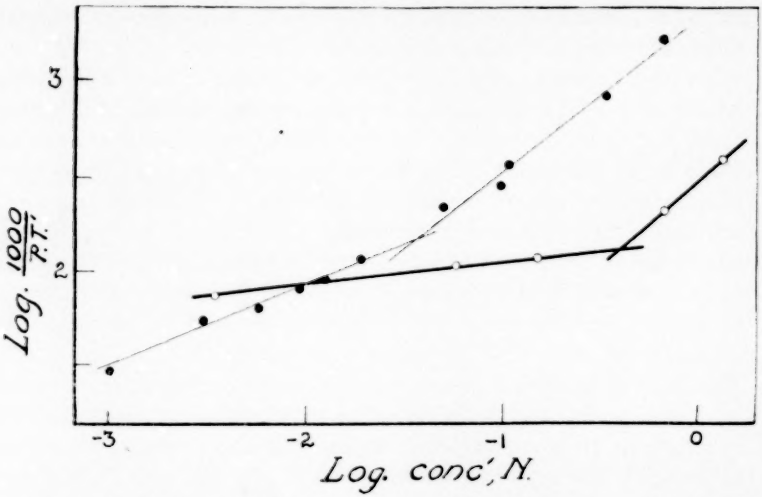

FIG. 3. The relation between speed of tissue penetration and acid concentration in solutions of HCl (light line) and H_3PO_4 (heavy line), when the formol concentration and apparent time of penetration have been corrected for the quantity of acid necessary to induce color change in the indicator system studied (27.0°).

esses. This general result is also obtained with other acids. Each part of these plots follows an equation of the form

$$\log\left(\frac{1}{P.\ T.'}\right) = \frac{1}{n}\log\ (\text{concentration}') - \log k$$

(*P. T.*, observed penetration time, *P. T.'*, corrected penetration time, k a constant), which is similar to the Freundlich formula for the adsorption isotherm and may in fact be derived from this formula by the aid of assumptions such as are commonly made in applying it. The curves shown in Fig. 3 have, however, no probable reference to adsorption; what they do express is the relation between the concentration of acid external to the tissue and its ability to force through the tissue a quantity of acid sufficient to induce the intracellular color change. With H_3PO_4 this ability increases very slowly up to a point where the osmotic pressure of the acid is adequate rapidly to overcome the resistance to its diffusion, which is produced in the tissue by the acid itself; beyond

this point the inward diffusion of acid is very rapid, and the time for the observed effect is nearly proportional to the dilution of the acid. It may be stated here that the temperature coefficient of penetration speed, for this latter portion of the curves, is of the order $Q_{10} = 2.0$, indicating the chemical nature of the principal factor governing the penetration (as might be expected). The coagulative action of H_3PO_4 is so great that the resistance to subsequent diffusion is overcome only very slowly until a high concentration of the acid is used. From these facts it may be inferred that the primary action of the H_3PO_4 is more intense than that, *e.g.*, of HCl (at equal concentration). If comparison is made at a point in the reduced data (Fig. 3) where the $C_H = 0.01$ N, it will be noticed that the H_3PO_4 is slightly more active than HCl. These considerations explain why the apparent speed of penetration of H_3PO_4 from higher concentrations gives a more correct idea of the primary effect of this acid upon the tissue used; this is not the case with some other acids.

SUMMARY.

V. The relative apparent speed with which H_3PO_4 penetrates different tissues is conspicuously modified by the density of the cells concerned, and by the proportion of "buffer" substances present. When the gross observations of apparent penetration time are corrected by consideration of the limiting concentration of acid inducing visible color change, it is found that the observations for each acid fall upon two intersecting curves of the form:

$$\frac{1}{P.\,T.'} = k\ (\text{concentration}')^{\frac{1}{n}}$$

which does not, however, have reference to adsorption. With mantle tissue of *Chromodoris* the coagulative action of H_3PO_4 is so pronounced that a relatively high concentration must be used before there is marked acceleration in the speed with which the quantity of acid sufficient to react with the indicator is forced to diffuse into the tissue. The primary (surface) action of H_3PO_4 is greater than is made evident by its apparent penetrating power, and is also (probably) somewhat greater than its hydrogen ionization would demand.

AGAR'S ISLAND, BERMUDA.

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STUDIES OF EXPERIMENTAL SCURVY.

II. THE INFLUENCE OF GRAINS, OTHER THAN OATS, AND SPECIFIC CARBOHYDRATES ON THE DEVELOPMENT OF SCURVY.*

By W. PITZ.

(From the Department of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, January 29, 1918.)

In a recent publication from this laboratory McCollum and Pitz (1) put forth the theory that scurvy in the guinea pig is not the result of a deficiency of any specific protective substance, but that it is the result of the absorption of toxic substances arising from putrefaction in the cecum due to an undue retention of feces. In the present paper further proof is given in substantiation of this theory.

Hull and Rettger (2) showed with white rats and also with men fed on a high protein diet that the bacterial flora of the intestine could be changed from a putrefactive flora to an acidophilus flora by the ingestion of lactose in the diet. Other carbohydrates as sucrose, dextrose, maltose, dextrin, levulose, and galactose, produced some change in the intestinal flora of the rat but the change was not nearly so pronounced as in the case of the lactose. When lactose was fed with a high protein diet the flora was almost completely changed to the acidophilus type with but few or no evidences of putrefactive bacteria. With the other carbohydrates used, both types of bacteria were found but the number of putrefactive organisms was decreased and the number of acidophilus organisms increased as compared with the flora of an animal receiving a high protein diet, without the addition of a carbohydrate.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

If scurvy is the result of the absorption of putrefactive products arising from the putrefaction of feces in the cecum, then any substance which will improve or change the intestinal flora from a putrefactive to an acidophilus type should prevent the onset of the symptoms of scurvy. In order to test this supposition various groups of guinea pigs were fed oat meal and whole milk, plus a carbohydrate.

Chart 1 shows the remarkable recovery of a guinea pig when fed lactose after it had shown signs of very severe scurvy. The animal had swollen joints and was able to move about only with great difficulty. It was very sensitive and cried when touched. When 20 per cent of lactose was added to the oats the condition of the animal was bettered in a week. It gained in weight and improved rapidly. In 2 weeks it was able to walk about with ease and it soon became very active. The knees always remained a little swollen, but otherwise the animal appeared normal in all respects. When the animal was killed at the end of the 19th week, it showed no symptoms of scurvy except for the slight swelling of the knees. There were no hemorrhages at the knees or at the costochondral joints of the ribs.

Chart 2 demonstrates the protective action of lactose in a ration of rolled oats and milk. The animals lost weight for about 3 weeks, but at no time did they show any evidence that they were suffering from scurvy. After the 3rd week the animals gained in weight, were vigorous, very sleek in appearance, and very active. Animal 2 lost weight after the 12th week and died at the end of the 18th week. Postmortem examination failed to reveal any of the characteristic symptoms of scurvy or the cause of death. The other animals were killed at the end of the 20th week and a postmortem examination was made on each one. They were all in excellent condition, and as far as I was able to determine they were normal in all respects. The lactose not only must have changed the intestinal flora of the tract as can be assumed from the work of Rettger, but in the amounts used it acted to some degree as a laxative. Since milk was fed *ad libitum* it cannot be argued that any specific substance was added with the lactose which was not already present in considerable amounts.

Chart 3 demonstrates that although 10 per cent of lactose was beneficial to some degree it was not sufficient to protect the ani-

mals from scurvy for more than a few weeks when fed rolled oats and milk.

Charts 4, 5, 6, and 7 show the effect of feeding dextrin, dextrose, sucrose, and starch with the standard ration of rolled oats and whole milk. In every case some protection was afforded, although in the case of dextrose the protection was but slight. The appearance of these animals was better than that of animals receiving only oats and milk, but their appearance was not nearly so good as that of the animals shown in Chart 2. The growth curves show considerable improvement over the growth curves of animals receiving no additional carbohydrate (compare Charts 4, 5, 6, and 7 with Chart 8). This slight protection afforded by dextrin, dextrose, sucrose, and starch is explained by the findings of Hull and Rettger as cited above and also by the work of Hirschler (3). The latter showed that, *in vitro*, sucrose, glycerol, dextrin, starch, and lactic acid completely prevented the formation of indole, scatole, and phenol over a period of 10 days, from proteins which were inoculated with cultures of putrefactive organisms. In the animal body the same carbohydrates did not completely prevent the formation of indole, scatole, and phenol in the feces of dogs fed a high protein diet, but they did materially decrease the amounts of these products formed as compared with the amounts found in the feces of dogs fed a high meat diet but receiving no carbohydrates.

These results demonstrate beyond a doubt that a putrefactive flora in the intestine of the animal is instrumental in the production of scurvy, and that a diet which will cause a change in the flora from a putrefactive type to an acidophilus type will protect the animal from scurvy.

Production of Scurvy by Grains Other than Oats.

Lusk (4) makes the following statement, "It is important to remember that it has never been demonstrated that any kind of unmilled grain will produce scurvy." If scurvy is the result of the retention of feces, and there can be little doubt that such is the case, then any ration which will produce feces difficult to eliminate should produce scurvy. It has long been known that milk when taken in large quantities will cause severe constipa-

tion. This being true, then any grain as barley, wheat, corn (maize), etc., when fed with an abundant supply of milk should produce scurvy in the guinea pig. To test this point whole corn, wheat, and barley were ground, and the ground grains fed to guinea pigs together with all the milk they would consume.

Charts 9, 10, and 11 show the results of these experiments. It will be noted that these curves show decidedly better growth than do the curves of guinea pigs fed on whole oats and milk (see Chart 12). The animals were in better condition and did not show symptoms of scurvy as soon as did oat-fed animals. The mortality of the animals receiving corn, wheat, or barley was very high, and postmortem examination of the animals that died showed the characteristic lesions of scurvy in all but a few cases. One animal on the barley and milk ration was still alive at the end of 18 weeks. This animal shows no evidence of having scurvy at the present time. From the 4th week to the 8th week, however, it was in very poor condition and showed signs of scurvy, but after the 8th week it slowly improved and at the present time is in excellent condition. This ability of an animal that has scurvy to slowly recover without the administration of any remedial agents has been observed at various times. It occurs on different rations and is probably due to unusual vigor and resistance of the individual.

Two animals are still alive on the corn and milk ration, but although they are gaining in weight they both have swollen knee joints and are very sensitive. The coats are rough and the animals appear to be poorly nourished, in spite of the fact that they are gaining in weight. This ability of a scurvy animal to gain in weight has been noted by Jackson and Moore (5) in their work with guinea pigs.

Two animals are alive on the wheat and milk diet at the time of writing this paper. One of these, No. 2, shows no symptoms of scurvy although it has been losing weight for the past 4 weeks. The other animal, No. 4, has swollen knee joints, walks with considerable difficulty, and shows signs of severe scurvy.

The lower efficiency of rolled oats and whole oats as compared with other grains in the nutrition of the animal is due in part to the peculiar property of oats to produce very pasty feces which are difficult to eliminate. This lower efficiency of the oat

kernel, due to its formation of pasty feces, when fed rats was also noted by McCollum, Simmonds, and Pitz (6), in their investigation of the dietary deficiencies of the oat kernel.

Animals that received corn, wheat, or barley and milk were protected from scurvy by the addition of lactose to the ration. With these grains, however, 10 per cent of lactose when added to the grain was just as effective in preventing scurvy as was 20 per cent of lactose when fed with oats. 20 per cent of lactose when fed with corn, wheat, or barley caused abnormal fermentation in the tract of the animal and caused the abdomen to be greatly distended with gas.

These results show that scurvy can be produced in the guinea pig by feeding a ration of unmilled grains and milk.

I wish to thank Professor E. B. Hart for the many suggestions which he offered and especially for his suggestion of the feeding of lactose and the various grains.

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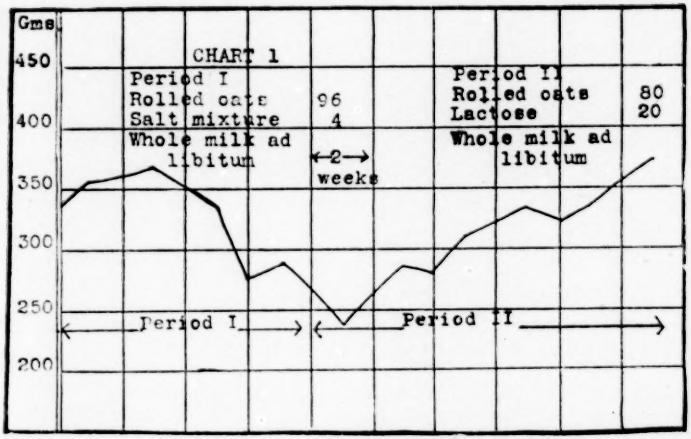

CHART 1. This chart illustrates the remarkable recovery of an animal when fed lactose in the ration. During the first period the animal received a ration consisting of oat meal and a salt mixture and whole milk. The salt mixture consisted of inorganic elements in the same proportions as found in orange juice, but acetic acid was put into the mixture in place of citric acid. It was shown in our earlier paper that this same salt mixture, but containing citric instead of acetic acid, prevented the development of scurvy in a diet of rolled oats and heated carrots. During the second period the animal was given a ration consisting of rolled oats 80 and lactose 20, plus whole milk. At the beginning of the lactose feeding the animal was in a most miserable condition. It was very weak, moved about with great difficulty, and was very sensitive. After the lactose was introduced into the ration the animal gained in weight rapidly and showed marked improvement. All symptoms of scurvy disappeared except for a slight swelling at the knee joints. Postmortem examination failed to reveal the characteristic lesions of scurvy.

Composition of salt mixture.

	gm.
Ca citrate	2.261
$MgCl_2 6H_2O$	1.270
K citrate	2.578
NaCl	0.787
K_2HPO_4	0.837
Acetic acid	0.960
Sucrose	8.270

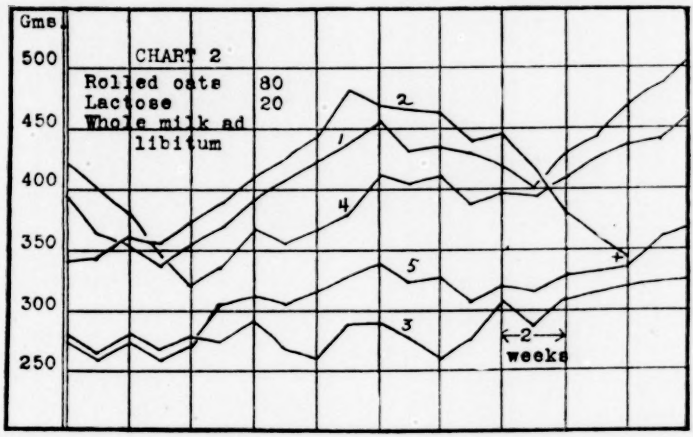

CHART 2. This record gives further evidence of the beneficial effects resulting from the ingestion of 20 per cent of lactose with an oat and milk diet (compare with Chart 8). All the animals except No. 2 were in excellent condition throughout the experiment. Animal 2 was in fine condition until the 12th week. At this time the animal began to lose weight and became thin and inactive, but it did not show any lesions of scurvy either when alive or on postmortem examination.

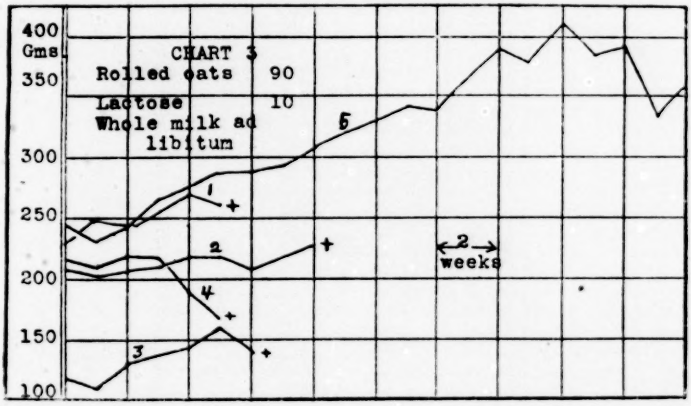

CHART 3. These curves show that 10 per cent of lactose was not as efficient in protecting the animals from scurvy as was 20 per cent. Postmortem examination of Animals 1, 2, 3, and 4 showed lesions of scurvy. Animal 5 grew fairly well throughout the course of the experiment and never showed any signs of having scurvy. It was killed at the end of the 20th week and appeared to be normal in all respects.

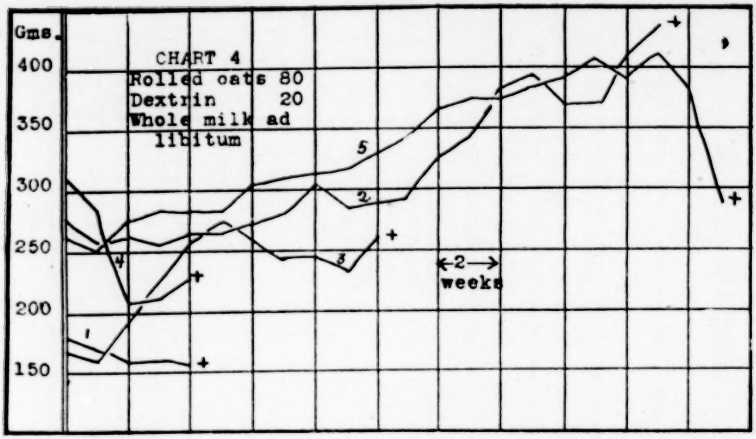

CHART 4. This chart shows the record of animals fed on a ration of rolled oats and dextrin prepared from cornstarch. It is evident by comparing these curves with those of Chart 8 that these animals were benefited to a considerable degree by the ingestion of dextrin in the ration, but they did not do nearly so well as did the animals that received lactose (see Chart 2). All the animals died, but there was a marked difference in the length of time that the animals survived this ration. Animals 2 and 5, which survived the longest, showed the most severe lesions of scurvy. Animal 1 did not show symptoms of scurvy on postmortem examination.

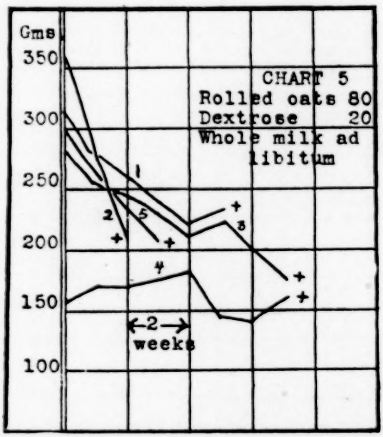

CHART 5. These animals received dextrose in the ration. They did little better than the animals that received no carbohydrate, other than that contained in the oats (see Chart 8). The animals showed severe lesions of scurvy. Two of them, Nos. 4 and 5, suffered with an unusual amount of fermentation. The cecum of all of the animals on this diet was packed with feces that had a very offensive odor.

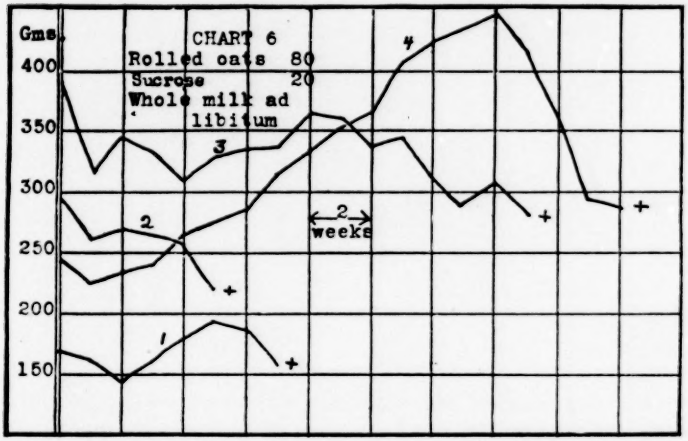

CHART 6. This chart illustrates the behavior of guinea pigs fed on a diet of rolled oats and sucrose and milk. The ingestion of sucrose delayed the onset of the symptoms of scurvy for a considerable time, but eventually the animals all died of scurvy.

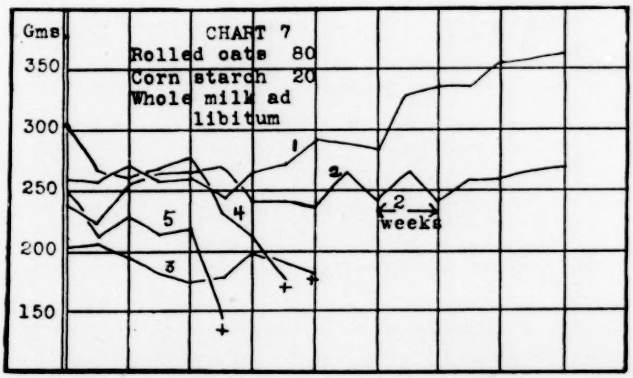

CHART 7. These animals received a ration of rolled oats and cornstarch. The starch afforded considerable protection against scurvy. However, three animals died of scurvy before the 9th week and all showed lesions at this time. Animal 1 is alive at the present writing and is in fair condition. It, however, shows no outward signs of scurvy. Animal 2 shows distinct signs of scurvy, but it is maintaining its weight.

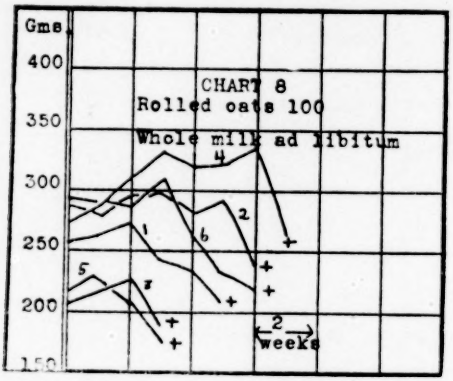

CHART 8. This chart illustrates the typical behavior of guinea pigs fed a ration of rolled oats and whole milk *ad libitum*. The animals generally develop the lesions of scurvy from the 3rd to the 4th week, but a few live longer than this before they show the symptoms. Occasionally an exceptional individual will be found that thrives on this ration. The curve of such an animal was given in our first paper on scurvy (1).

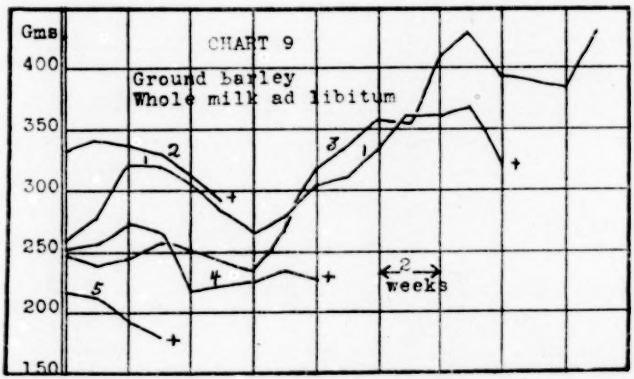

CHART 9. This illustrates the behavior of guinea pigs fed a ration of ground barley and whole milk *ad libitum*. The animals thrive better on this ration than they do on a ration of whole ground oats and milk (see Chart 12). All the animals on this ration, except No. 3, showed signs of scurvy about the 4th week. Animal 3 did not show lesions of scurvy at any time and is in excellent condition at this writing. It should be borne in mind that occasionally a guinea pig will thrive on an oat and milk diet.

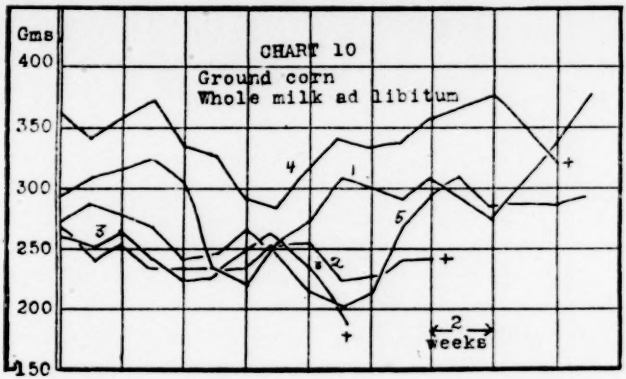

CHART 10. Growth curves of animals fed a ration of corn and milk. The animals did not lose weight as rapidly and they lived longer than did animals receiving barley and milk (see Chart 9). The symptoms of scurvy were not evident in these animals until the 5th and 6th weeks. Animal 5 did not show lesions until the 8th week. Animals 1 and 5 are alive at the present writing. No. 1 shows symptoms of mild scurvy, while No. 5 shows more pronounced symptoms.

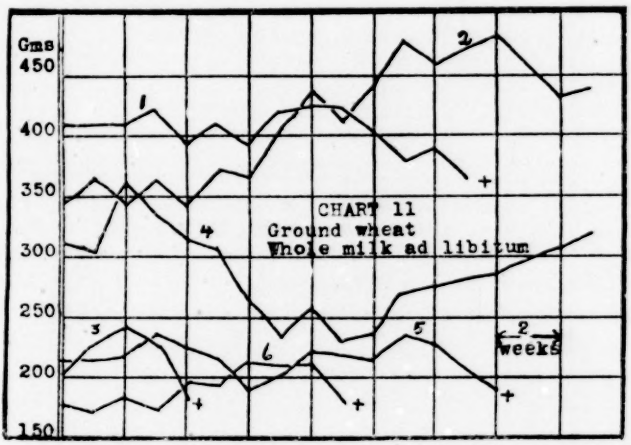

CHART 11. The animals in this group received a ration of whole wheat and milk. These animals grew about as well as did the animals that received corn and milk (see Chart 10). However, the animals in this lot developed lesions of scurvy during the 3rd and 4th weeks, while those on corn developed them the 5th to 6th weeks. Animal 2 never showed any evidence of having scurvy and it remained in excellent condition until the 12th week. Since then it has been losing weight, but it does not show any signs of having scurvy at the present time. Animal 4 developed scurvy the 4th week, and from the 4th to the 8th week it was in a miserable condition. After the 8th week it slowly improved, but its knee joints are still swollen and it walks with difficulty and is very sensitive.

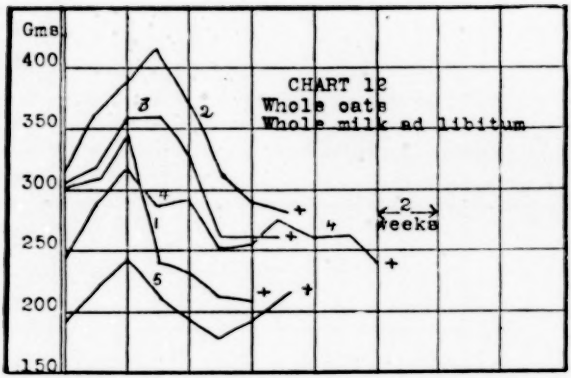

CHART 12. Behavior of animals fed a ration of whole oats and milk All these animals developed scurvy during the 3rd and 4th week. They failed rapidly and all but two died at the end of the 7th week. One animal lived until the 10th week.

A CHEMICAL STUDY OF FOOD FISHES.

THE ANALYSIS OF TWENTY COMMON FOOD FISHES WITH ESPECIAL REFERENCE TO A SEASONAL VARIATION IN COMPOSITION.

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That a variation in the composition of fish might occur was recognized by Atwater (1) who analyzed fifty-two species, sometimes basing his results on a single specimen, again taking the average of several samples caught at short intervals. Nine species were examined at different seasons of the year. Of these only three species—blackfish, mackerel, and salmon—showed a definite tendency toward a seasonal variation in composition.

A study of the recorded analyses of fish makes it plain that a single examination, even if based on large numbers of individuals, cannot be accepted as representing a typical composition. For example, the herring shows a wide latitude in its gross composition, as may be seen from Table I, where the moist fish ranges from 19.29 to 30.97 per cent of solids, and from 2.41 to 11.01 per cent of fat. This variation in the composition

TABLE I.

The Percentage Composition of Herring, as Determined by Various Investigators.

Fish.	Author.	Water.	Solids.	Fat.	Ash.	Fat-ash-free solids.	Nitrogen.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Fresh herring.	(See Table IV).	77.10	22.90	2.41	1.66	18.83	3.03
" "	Kostytscheff (3).	76.11	23.89	4.89	1.71	—	—
Little "	Almen (4).	73.22	26.75	5.87	1.65	18.83	3.01
Herring.	Buckland (5).	80.71	19.29	7.11	2.07	—	—
Smoked herring.	König (6).	69.49	30.51	8.51	1.24	21.12	3.38
Fresh "	Payen (7).	70.00	30.00	10.30	1.90	—	—
" "	Atwater (1).	69.03	30.97	11.01	1.50	19.12	3.06

of herring has been studied by Hjort (2). It is obvious, therefore, that we should know the locality of the catch, the season of the year, the weight of the fish, and also as much of its habits at the catching time as possible, in order to interpret analytical results.

Polimanti (8) has considered the relation of habitat to the fat content of the flesh and is of the opinion that the deep-sea fish are poor in fat while the surface swimming forms are rich. Aside from the relation between habitat and the content of fat, there is undoubtedly a close relation between the amount of fat stored in the edible portions of the fish and the processes of reproduction. This has been observed by Atwater (1), who gives the composition of ordinary and spent salmon. The fish on their way to the spawning grounds contained 13 per cent of fat, while the spent fish contained but 3 per cent. The work of Miescher-Ruesch (9) on the Rhine salmon gives a similar picture, and the later work of Paton (10) on Scotch salmon, and of Greene (11) on Pacific coast salmon, is confirmatory. According to the latter two investigators, the fat stored in the body of the salmon during the period of feeding and growth is the immediate source of energy expended by the fish during spawning migration when no food is taken. Also, the observation by Lichtenfelt (12), who noted that during hunger a fat-rich fish loses a greater proportion of its fat than a fat-poor fish, may, when the analytical data are supplied, throw some light upon the habits of certain species.

Whatever the biological significance of the changing composition may be, the fact remains that a large number of our food fishes have a greatly increased fat content in the late summer and autumn, as shown in Table II, compiled from Atwater's (1) analyses, and also by the analytical results recorded in Table IV.

TABLE II.

Analyses of Food Fish by Atwater, Arranged to Show Seasonal Variation. Fresh Basis.

Fish.		Water.	Solids.	Fat.	Ash.	Nitrogen.
Name.	When caught.					
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Blackfish.	Mar., 1882	81.36	18.64	0.55	0.65	2.82
"	Apr., 1882	79.64	20.36	0.62	1.03	3.03
"	May, 1882	78.44	21.56	1.44	1.36	3.03
Mackerel.	May, 1880	74.14	25.86	6.99	1.30	2.91
"	Dec., 1880	64.01	35.99	16.30	1.48	3.05

The results of Atwater's investigations have been accepted as representing the correct composition of American food fishes. The dietitian, in calculating the fuel value of the food components present in different species of fish, has used these figures. They have been quoted by a number of writers.

In addition to Atwater's classical work on American fishes, the literature contains numerous references bearing on the chemical composition of food fish. The earlier investigations have been carried on mainly by foreign workers, among whom are Schlossberger (13), Morin (14), Weidenbusch (15), von Baumhauer (16), Payen (7), König (6), Buckland (5), Almen (4), Kostytscheff (3), Popoff (17), Sempolowski (18), and Kianizyn (19). The results obtained by most of these authors have been discussed at length by Atwater (1). Their analyses consisted, generally, of determinations of solids, fat, ash, and nitrogen. There has been no concerted analytical study of the composition of American food fish since 1888. European food fishes have been studied more recently, and among the investigations those of Balland (20) and Hollande (21) are especially noteworthy. A comparison of the food value of beef, veal, etc., with fish has been made by Milone (22), Beythien (23), and König and Splittgerber (24).

Methods.

History of Samples.

The fish were all obtained from Philadelphia fish dealers. No fish were purchased of which an accurate history could not be obtained. This history indicated good handling according to present commercial standards and a minimum lapse of time after catching. The analytical work was begun immediately upon the receipt of the sample.

Preparation of Sample for Analysis.

The fish were cleaned by separating as completely as possible from the edible portion the head, tail, skin, entrails, and bones. The edible portion was weighed and the difference between this weight and that of the whole fish represented the refuse, or inedible portion. The flesh was ground in a meat chopper, and the individual samples were mixed thoroughly by hand. As the subsequent analysis requires 2 or 3 days of one analyst's time for completion, it was not convenient to undertake more than one complete analysis of a single species of fish at a time. In the case of large fish but one specimen was used, but of the smaller fish from 2 to 8 were analyzed, the edible portions being united and well mixed before sampling.

Methods of Analysis.

Total Solids, Total Nitrogen, Fat, and Ash.—The methods used for the estimation of the quantity of total solids, fat, and ash, were those given in Bulletin No. 107 (revised) of the Bureau of Chemistry, United States Department of Agriculture. Total nitrogen was determined by the method of Kjeldahl and Gunning, using 0.1 N solutions of acid and alkali.

Cold Water-Soluble Nitrogen.—The amount of nitrogenous compounds soluble in cold water was found by grinding 50 gm. of the sample, placing in a 12 ounce wide-necked bottle, adding 240 cc. of water, corking tightly, and shaking on a mechanical shaker. The bottles were removed from the shaker at the end of 15 minutes and the suspended matter was separated by centrifuging for 10 minutes. The opalescent supernatant fluid was poured through a plug of cotton in a funnel. Seven more extractions, each with 240 cc. of water, brought the final volume of the combined filtrate to about 1,900 cc. This solution was transferred to a 2 liter flask and diluted to the mark with water. A second filtration through paper was made when required, though this was usually unnecessary, as the extract could be obtained in most cases free from suspended matter. Aliquots of 50 cc. each were analyzed for nitrogen by the Kjeldahl-Gunning method.

Coagulable Nitrogen.—Portions of 500 cc. of the neutral extract were evaporated to a little less than 250 cc. in 400 cc. beakers. The beakers were placed on wide asbestos gauzes to prevent scorching, and the height of the flame was so regulated that about 2 hours were required to reduce the volume of the solution one-half. The mixture was stirred occasionally to prevent clumps from sticking to the bottom of the beaker. After removing the flame, and while the solutions were still hot, they were always treated with exactly 2 cc. of 20 per cent acetic acid solution to complete the precipitation of the coagulable protein. On cooling, the mixture was transferred to a 250 cc. volumetric flask and diluted to the mark with water. The mixture was filtered and aliquots of 25 cc. each were analyzed for nitrogen.

Hot Water-Soluble Nitrogen.—The flesh residue remaining from the cold water extraction was washed into a 400 cc. beaker with about 250 cc. of hot water. The mixture was boiled for 15 minutes, filtered hot through cotton, the residue again treated with hot water, and boiled for another 15 minutes. The filtrate from the second extraction was added to that of the first and the combined filtrates were transferred to a 500 cc. volumetric flask, cooled, and diluted to the mark with water. This extract was filtered through fine filter paper and nitrogen was determined on 50 cc. aliquots of the filtrate.

Ammonia Nitrogen.—For this determination a modification of the Steel-Gies[1] method was used. 10 gm. of NaCl were dissolved in 100 cc. of water

[1] Steel, M., and Gies, W. J., Some notes on the efficiency of the Folin method for the quantitative determination of urinary ammonia, *J. Biol.*

in a tall Folin aeration cylinder. 25 gm. of ground flesh were added and the mixture was shaken until the material was entirely disintegrated. 50 cc. of alcohol were next added to prevent foaming, and 5 cc. of kerosene later if necessary. The mixture was finally treated just previous to aeration with 10 cc. of 10 per cent sodium hydroxide solution. The ammonia was absorbed in dilute sulfuric acid solution (10 cc. of 0.1 N sulfuric acid in 100 cc. of water) in 12 ounce bottles. At the end of a 4½ hour aeration period, the solutions in the absorbing bottle were titrated back with 0.1 N sodium hydroxide solution, using as indicator two drops of 1 per cent alcoholic solution of alizarin red.

Gross Fat Extraction.

In order to obtain the fish oil in a condition as nearly unchanged as possible, care was constantly exercised to prevent overheating of the material. The ground flesh was placed in 6 inch shallow Petri dishes in sufficient quantity to form thin layers when pressed down on the bottoms of the dishes with a spatula. The water was driven off at 50°C. in a vacuum oven. The dried residue was ground, placed in a large paper extraction thimble, and extracted with low boiling petroleum ether (40–50°C.) in a Soxhlet extractor. After a 12 hour extraction, the solvent was evaporated somewhat, the mixture filtered through fine filter paper, the remainder of the ether carefully evaporated, and the oil finally dried for 3 hours at 50°C. in a vacuum oven.

Determination of Fat Constants.

In some fish the amount of fat obtained was so small that but very few of the fat constants could be determined, due to lack of material, and when such a condition existed usually only the iodine number was determined.

Specific Gravity.—The specific gravity was determined in a Sprengel or Ostwald pyknometer at 25°C., compared with water at the same temperature, and the constants were based on weights in air.

Index of Refraction.—This was determined at 30°C. with an Abbe refractometer.

Chem., 1908, v, 71. Steel, M., An improvement of the Folin method for the determination of urinary ammonia nitrogen, *ibid.*, 1910, viii, 365. Shulansky, J., and Gies, W. J., Studies of aeration methods for the determination of ammonium nitrogen. The ammonium nitrogen in beef, *Biochem. Bull.*, 1913, iii, 45. Clark, E. D., Report on meat and fish. 1915. *J. Assn. Agric. Chem.*, 1917, ii, 229.

TABLE III.

History of Samples of Fish Analyzed.

No. of sample.	Fish.	No. of fish analyzed.	Total weight.	When caught	Where caught.	How caught.	How shipped.	Edible portion.
			gm.	*1915*				*per cent*
19	Blackfish (*Tautoga onitis*).	1	3,432	June 16	Lavalette, N. J.	Traps.	Freight, in barrels, iced.	35.1
18	Bonita (*Sarda sarda*).	1	1,833	" 12	Sea Isle City, N. J.	"	" iced.	58.4
33	Cod (*Gadus callarias*).	1	2,475	Nov. 24	Holly Beach, N. J.	Hook.	" in boxes, iced.	49.9
16	Herring (*Clupea harengus*).	8	2,285	June 12	Asbury Park, N. J.	Traps.	" " barrels, iced.	50.5
8	Kingfish (*Menticirrhus americanus*).	3	1,782	May 4	Ocean City, Md.	"	" " " "	46.2
32	Ling (*Urophycis*, sp. (?)).	4	2,339	Nov. 18	Seaside Park, N. J.	Seine.	" " boxes, iced.	42.2
11	Porgy (*Stenotomus chrysops*).	7	2,029	May 14	Block Island, R. I.	"	" " barrels, iced.	37.1
34	Tilefish (*Lopholatilus chamaeonticeps*).	1	5,800	Dec. 17	150 miles off New Jersey shore.	Hook.	" " " "	50.8
14	Silver hake (*Merluccius bilinearis*).	6	2,720	May 26	Lake Como, N. J.	Traps.	" " " "	41.7
9	Bluefish (*Pomatomus saltatrix*).	1	2,769	May 7	Cape Charles, Va.	Hook.	Freight, in barrels, iced.	48.7
27	" " "	1	2,277	Sept. 28	Atlantic Highlands, N. J.	"	" " boxes, iced.	53.7
12	Butterfish (*Poronotus triacanthus*).	7	1,243	May 19	Sea Isle City, N. J.	Traps.	" " " "	44.2
28	" " "	8	1,938	Oct. 12	" " " " "	"	" " " "	52.7
15	Carp sucker (*Carpiodes thompsonii*).	1	1,683	May 29	Sandusky, Ohio (Lake Erie).	"	Express, " " "	33.9
30	" " (" *cyprinus*).	1	4,599	Oct. 20	Delaware Bay.	"	Fish were alive, no ice.	44.1
3	Croaker (*Micropogon undulatus*).	6	1,677	Apr. 10	Buckroe Beach, Va.	Seine.	Freight, in barrels, iced.	30.9
23	" " "	8	1,703	Sept. 8	Chincoteague Island, Va.	Trawl.	" " " "	37.8
6	Flounder (*Pseudopleuronectes americanus*).	2	2,076	Apr. 19	Vineyard Sound.	"	" " " "	34.9
25	" " "	1	2,275	Sept. 22	Long Branch, N. J.	Seine.	" " boxes, iced.	48.4

2	Haddock (*Melanogrammus æglefinus*).	2	3,625	Apr. 2	Georges Bank, Mass.	Hook.	Freight, in boxes, iced.		42.9
22	" " "	1	1,365	Aug. 31	" " "	"	" " " "		52.1
5	Striped bass (*Roccus lineatus*).	3	2,775	Apr. 16	Wanchese, N. C.	Gill net.	" " " "		38.7
29	" " " "	7	2,469	Oct. 16	Potomac River.	Hook.	Express, " " "		43.6
10	Sea bass (*Centropristes striatus*).	2	2,562	May 12	Cape May, N. J.	"	" " " "		34.5
24	" " " "	5	1,657	Sept. 14	Anglesea, N. J.	"	" " " "		37.7
17	Spanish mackerel (*Scomberomorus maculatus*).	3	2,658	June 4	Off Boston, Mass.	Seine.	Freight, " barrels, iced.		51.7
31	" " " "	2	2,667	Oct. 26	Race Point, Mass.	"	" " boxes, "		62.7
7	Weakfish (*Cynoscion regalis*).	3	1,339	May 1	Ocean City, Md.	Traps.	Express, " barrels, "		43.8
26	" " "	3	1,922	Sept. 25	Long Branch, N. J.	Seine.	Freight, " " "		54.1
1	Shad (*Alosa sapidissima*) (male).	2	3,988	Apr. 2	Albemarle Sound, Wanchese, N. C.	Seine.	Freight, in boxes, iced.		51.6
4	" " " (female).	1	2,627	" 13	Wanchese, N. C.	"	" " " "		47.8
13	" " " "	1	1,998	May 22	Port Penn, Del.	Traps.	" " " "		40.1
20	" " " " spent.	2	1,309	June 19	Ship Bottom, N. J.	"	" " barrels, iced.		52.8

TABLE IV.

Percentage Composition of Edible Part of Fish.
Fresh Basis.

No. of sample.	Common name of fish.	When caught.	Solids.	Fat.	Ash.	Nitrogen.				
						Total.*	Cold water-soluble.	Coagulable.	Hot water-soluble.	Ammonia.
		1915	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
19	Blackfish	June 16	20.0	0.15	1.40	2.93	0.874	0.532	0.241	0.0172
18	Bonita	" 12	26.66	1.46	1.71	3.82	1.030	0.389	0.088	0.0251
33	Cod	Nov. 24	18.65	0.09	1.23	2.95	0.950	0.530	0.330	0.0170
16	Herring	June 12	22.90	2.41	1.66	3.03	1.028	0.516	0.234	0.0177
8	Kingfish	May 4	24.60	5.24	1.39	2.83	0.838	0.425	0.172	0.0162
32	Ling	Nov. 18	18.30	0.12	1.15	2.69	1.030	0.650	0.240	0.0180
11	Porgy	May 14	23.39	2.59	1.37	3.02	1.001	0.620	0.221	0.0165
34	Tilefish	Dec. 17	19.66	0.51	1.35	2.80	0.770	0.380	0.220	0.0130
14	Silver hake	May 26	18.86	1.41	1.22	2.60	1.025	0.638	0.211	0.0140
9	Bluefish	May 7	23.83	1.54	1.16	3.36	0.760	0.320	0.071	0.0185
27	"	Sept. 28	29.04	8.10	1.11	3.26	0.730	0.360	0.085	0.0180
12	Butterfish	May 19	25.66	5.96	1.49	2.89	0.862	0.498	0.020	0.0162
28	"	Oct. 12	30.01	13.52	1.40	2.92	0.850	0.430	0.021	0.0180
15	Carp sucker	May 29	22.80	2.10	1.20	2.98	1.151	0.787	0.206	0.0154
30	" "	Oct. 20	24.79	4.17	1.20	3.19	1.078	0.748	0.210	0.0170
3	Croaker	Apr. 10	20.77	1.25	1.18	2.85	0.813	0.447	0.141	0.0140
23	"	Sept. 8	24.26	3.23	1.37	—	0.880	0.410	0.130	0.0230
6	Flounder	Apr. 19	17.54	0.20	1.17	2.56	0.888	0.477	0.117	0.0143
25	"	Sept. 22	21.59	0.37	1.34	2.54	0.740	0.330	0.200	0.0190
2	Haddock	Apr. 2	18.32	0.15	1.11	2.33	0.748	0.343	0.147	0.0220
22	"	Aug. 31	20.83	0.09	1.01	2.59	0.800	0.520	0.150	0.0220
5	Striped bass	Apr. 16	25.70	3.58	1.26	3.21	1.153	0.719	0.102	0.0170
29	" "	Oct. 16	19.83	2.98	1.26	3.07	1.050	0.650	—	0.0166
10	Sea bass	May 12	22.02	1.61	1.23	2.98	0.967	0.532	0.106	0.0159
24	" "	Sept. 14	19.44	1.60	1.09	—	0.970	0.700	—	0.0220
17	Spanish mackerel	June 4	33.01	12.59	1.20	3.13	0.846	0.458	0.055	0.0185
31	" "	Oct. 26	35.70	16.24	1.11	3.09	0.890	0.570	0.102	0.0240
7	Weakfish	May 1	21.41	2.34	1.25	2.83	1.118	0.851	0.203	0.0134
26	"	Sept. 25	19.35	0.52	1.20	—	0.820	0.500	0.290	0.0150
1	Shad (male)	Apr. 2	35.32	14.43	1.34	3.18	1.112	0.621	0.074	0.0160
4	" (female)	" 13	34.17	13.93	1.40	3.00	1.147	0.685	0.063	0.0182
13	" "	May 22	26.00	5.87	1.29	2.91	0.980	0.549	0.058	0.0191
20	" " spent	June 19	23.38	2.95	1.53	2.98	0.975	0.549	0.182	0.0178

* It is customary to calculate protein as total nitrogen × 6.25. Our knowledge of the proper factor to use with fish proteins is unsatisfactory and pending investigation of this matter it seems best to omit this calculation.

TABLE V.

Percentage Composition of Edible Part of Fish.
Calculated on Dry Basis.

No. of sample.	Common name of fish.	When caught.	Fat.	Ash.	Nitrogen.				
					Total.	Cold water-soluble.	Coagulable	Hot water-soluble.	Ammonia.
		1915	per cent	per cent	per cent	per cent	per cent	per cent	per cent
19	Blackfish	June 16	0.70	6.70	14.02	4.18	2.55	1.15	0.082
18	Bonita	" 12	5.48	6.41	14.33	3.86	1.46	0.33	0.094
33	Cod	Nov. 24	0.46	6.59	15.81	5.09	2.84	1.76	0.091
16	Herring	June 12	10.52	7.25	13.23	4.49	2.25	1.02	0.078
8	Kingfish	May 4	21.32	5.65	11.50	3.41	1.73	0.70	0.066
32	Ling	Nov. 18	0.62	6.27	14.68	5.62	3.55	1.31	0.098
11	Porgy	May 14	11.10	5.85	12.92	4.28	2.67	0.95	0.071
34	Tilefish	Dec. 17	2.59	6.85	14.22	3.91	1.93	1.11	0.066
14	Silver hake	May 26	7.48	6.48	13.80	5.43	3.39	1.42	0.074
9	Bluefish	May 7	6.45	4.87	14.10	3.19	1.35	0.30	0.078
27	"	Sept. 28	27.83	3.81	11.21	2.51	1.24	0.29	0.061
12	Butterfish	May 19	23.27	5.81	11.28	3.36	1.94	0.08	0.063
28	"	Oct. 12	45.02	4.66	9.72	2.83	1.43	0.70	0.059
15	Carp sucker	May 29	9.20	5.27	13.08	5.05	3.45	0.90	0.068
30	" "	Oct. 20	18.05	5.19	13.81	4.35	3.02	0.90	0.074
3	Croaker	Apr. 10	6.02	5.68	13.72	3.91	2.15	0.68	0.067
23	"	Sept. 8	13.30	5.64	—	3.62	1.69	0.54	0.094
6	Flounder	Apr. 19	1.14	6.67	14.60	5.06	2.72	0.67	0.082
25	"	Sept. 22	1.71	6.20	11.76	3.42	1.90	0.90	0.087
2	Haddock	Apr. 2	0.81	6.06	12.72	4.08	1.87	0.80	0.120
22	"	Aug. 31	0.42	4.84	12.43	3.84	2.50	0.72	0.105
5	Striped bass	Apr. 16	13.94	4.90	12.50	4.48	2.80	0.40	0.066
29	" "	Oct. 16	15.01	6.35	15.47	5.39	3.29	—	0.084
10	Sea bass	May 12	7.32	5.59	13.55	4.39	2.42	0.48	0.072
24	" "	Sept. 14	8.22	5.60	—	5.08	3.55	—	0.113
17	Spanish mackerel	June 4	38.14	3.63	9.48	2.56	1.39	0.17	0.056
31	" "	Oct. 26	45.47	3.10	7.38	2.49	1.60	0.29	0.067
7	Weakfish	May 1	10.94	5.85	13.23	5.53	3.98	0.95	0.063
26	"	Sept. 25	2.68	6.19	—	4.63	2.58	1.49	0.077
1	Shad (male)	Apr. 2	40.85	3.80	9.01	3.15	1.768	0.21	0.045
4	" (female)	" 13	40.75	4.10	8.78	3.35	2.005	0.18	0.053
13	" "	May 22	22.58	4.97	11.20	3.77	2.113	0.23	0.074
20	" " spent	June 19	12.62	6.54	12.75	4.17	2.348	0.78	0.076

TABLE VI.

Fat Constants of Oils from Fish Flesh.

No. of sample.	Common name of fish.	When caught.	Specific gravity, $\frac{25°}{25°}$ C.	Index of refraction 30° C.	Iodine No.	Saponification No.	Acid value.
		1915					
19	Blackfish	June 16	—	—	127.5	—	—
18	Bonita	" 12	0.9389	1.4755	126.8	178.7	28.6
33	Cod	Nov. 24	—	—	94.1	—	—
16	Herring	June 12	0.9292	1.4733	135.4	180.9	13.5
8	Kingfish	May 4	0.9146	1.4663	85.4	195.7	3.4
32	Ling	Nov. 18	—	—	80.8	—	—
11	Porgy	May 14	0.9270	1.4736	136.2	188.4	7.2
34	Tilefish	Dec. 17	—	1.4753	118.8	—	—
14	Silver hake	May 26	0.9397	1.4797	159.0	186.5	10.1
9	Bluefish	May 7	0.9353	1.4749	118.9	185.1	12.4
27	"	Sept. 28	0.9389	1.4760	106.9	—	4.5
12	Butterfish	May 19	0.9213	1.4732	138.2	190.5	3.0
28	"	Oct. 12	0.9177	1.4706	90.2	202.8	—
15	Carp sucker	May 29	0.9232	1.4704	111.7	187.9	7.8
30	" "	Oct. 20	0.9243	1.4700	74.1	240.8	—
3	Croaker	Apr. 10	0.9312	1.4724	115.8	191.2	—
23	"	Sept. 8	—	1.4703	111.5	—	—
6	Flounder	Apr. 19	—	1.4935	130.4	—	—
25	"	Sept. 22	—	1.4820	118.9	197.6	—
2	Haddock	Apr. 2	—	1.4940	133.4	—	—
22	"	Aug. 31	—	1.4907	115.1	—	—
5	Striped bass	Apr. 16	0.9278	1.4748	134.4	196.1	6.4
29	" "	Oct. 16	—	1.4895	89.0	—	—
10	Sea bass	May 12	0.9224	1.4731	132.1	191.7	2.2
24	" "	Sept. 14	—	1.4860	132.2	—	—
17	Spanish mackerel	June 4	0.9201	1.4719	136.7	182.5	3.2
31	" "	Oct. 26	—	1.4730	115.3	204.9	2.6
7	Weakfish	May 1	0.9213	1.4717	120.8	187.0	12.2
26	"	Sept. 25	—	1.4830	104.37	188.4	16.2
1	Shad (male)	Apr. 2	0.9084	1.4709	114.8	191.6	4.6
4	" (female)	" 13	0.9150	1.4710	114.5	190.4	3.5
13	" "	May 22	0.9124	1.4719	121.9	183.7	6.4
20	" " spent	June 19	0.9276	1.4725	125.3	180.6	20.6

Iodine Number.—The iodine absorption number was determined by the Hanus Method[2] on 0.1 to 0.2 gm. samples using 25 cc. of iodine solution.

Saponification Number.—Approximately 1.5 gm. of oil were saponified in the usual manner[3] with 25 cc. of alcoholic potash for this determination.

Acid Value.—1 gm. of oil was dissolved in 50 cc. of a neutral mixture of three parts ethyl ether and seven parts 95 per cent alcohol, and titrated with 0.1 N sodium hydroxide solution, using 0.5 cc. of 0.5 per cent alcoholic phenolphthalein solution as indicator. The addition of alkali was continued until one drop produced a pink color which persisted at least 1 minute. The result was calculated to mg. of potassium hydroxide required to neutralize the free fatty acids of 1 gm. of the oil.[3]

Tabulation of Analyses.

It was not possible to obtain samples of each species at varying seasons, hence of some fish but one analysis appears in the tables. It is believed that, aside from their interest in the light of seasonal variation, such analytical data are of value from the composition viewpoint alone. Tables III, IV, V, and VI record the results obtained. The values given are in all cases the average of two or more concordant determinations.

DISCUSSION.

Biological Significance of the Fat Content.

Many sea fish travel in schools, or migrate, in response to stimuli of which we are still ignorant and to regions often unknown at definite times of the year. They feed on small animal organisms, lower plant forms, or other fish, depending upon the habits of the various species. In the contest with fellow members of the school, certain fish will fare better in feeding than the less strong and active ones. This may account for the fact that fish of the same size from the same school occasionally vary considerably in fat content. The fish in Table VII were taken from the same school and show an exceptional degree of variation in this respect.

[2] Hanus, J., Die Anwendung von Jodmonobromid bei der Analyse von Fetten und Oelen, *Z. Untersuch. Nahrungs.-Genussmittel*, 1901, iv, 913.

[3] Official and provisional methods of analysis, *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107* (rev.), 1909, 136.

TABLE VII.

Analyses of Weakfish from the Same School. (Caught November 11, 1914.) Composite Samples of Three Fish Each.

No. of sample.	Solids.	Fat.	Ash.	Total nitrogen.
	per cent	*per cent*	*per cent*	*per cent*
1	22.88	1.35	1.16	3.13
2	23.35	2.47	1.10	3.13
3	25.51	4.88	1.16	3.09
4	28.22	8.03	1.12	3.19

A study of Table IV shows that weakfish may contain more fat in the spring than in the early autumn. We know but little of the habits of the weakfish. However, during September we find them full of feed, although the fat content of the fish is extremely low; for example, 0.52 per cent. It may be deduced from this figure that the fish have recently entered upon a feeding period, which assumption is further confirmed by the fact that later in the year—about the middle of November—as much as 8.03 per cent of fat was found (Table VII).

The literature fails to record the analysis of the flesh of the shad before and after its spawning migration. Accordingly, a study of this fish was made, covering a period of about 2 months, during which time the fish had appeared at the mouth of the rivers and had ascended them to fresh water. The later analyses were made at the close of the shad season. The results are shown in Table IV. The most striking differences are those in the fat, this constituent decreasing from 14.43 per cent *before spawning* in April to 2.95 per cent *after spawning* in June. There is likewise a decrease in the water-soluble and coagulable nitrogen, an increase in the specific gravity, iodine number, and acid value of the fats, and a lowering of their saponification numbers.

The shad, like the salmon, does not feed from the time it enters upon spawning migration until it returns to the feeding grounds. Some of the fat is consumed in the tissues to furnish the energy necessary for ascending the rivers, and the remainder is probably transferred to the roe and milt. It would be profitable to extend this investigation to a similar study of the changes in other fish at spawning time.

A fundamental relation, which is indicated not only by these results but likewise by the results obtained by Atwater (1), is that as the fat content increases the water content decreases, the protein remaining practically the same. This is strikingly shown in Table VIII in four species of fish whose fat content varies from 0.15 per cent to 14.43 per cent. The fat-free solids, which consist of nitrogenous and mineral constituents, vary but slightly from the mean value of about 21 per cent, showing that the gain or loss in fat is compensated for by a corresponding gain or loss in water rather than protein.

TABLE VIII.

Relation between the Amounts of Fat, Water, and Nitrogen in the Flesh of Fish.

Kind of fish.	Water.	Solids.	Fat.	Fat-free solids.
	per cent	*per cent*	*per cent*	*per cent*
Blackfish	79.10	20.90	0.15	20.75
Bluefish	70.96	29.04	8.10	20.94
Spanish mackerel	66.99	33.01	12.59	20.42
Shad	64.68	35.32	14.43	20.89

A study of the values obtained for the fat constants of the fish oils shows that they are fairly uniform for the different species analyzed. These constants will average, as a rule, approximately as follows:

Specific gravity at $\frac{25}{25}$°C	0.9220
Index of refraction at 30°C	1.4750
Iodine No	110–130
Saponification No	180–190

They are characteristic of fish oils commonly classed with drying oils.

A study of the figures obtained for the acid value of the oils (Table VI) shows that, in general, when fish are low in percentage of fat, the acid value will be high, and when the fat content is high the acid value will be low. This seems to point to the presence in the tissues of fish, in a more or less constant proportion, regardless of the fat content, of an acid substance sol-

uble in the fat solvent, or carried out with the fat. Accordingly, when the fat content of the fish is high the acid value is low, because the proportion of this substance to the total fat is low.

The Significance of the Variation in the Distribution of Nitrogen of the Flesh.

An examination of the nitrogen distribution, as given in Table IV, brings out certain facts. It will be observed that not only was the amount of nitrogenous substance soluble in cold water determined, but also the amount extracted by boiling water. The latter solvent was used because of the likelihood of relatively large, and possibly significant, quantities of gelatin-like substances in fish tissues. The results indicate that the amount is more or less constant for fish of the same species but may vary with difference in species. This trend is much more clearly shown in the amount of nitrogenous substances soluble in cold water, where it is seen that the amount of water-soluble nitrogen and coagulable nitrogen is fairly constant for the same fish taken at different times, while the amounts of these substances may be widely different for different species of fish.

The quantity of nitrogen obtained by aeration is generally accepted as indicating the progress of postmortem changes in the composition of flesh, especially those of decay as opposed to ripening changes. The quantities of ammoniacal nitrogen obtained from fresh fish of different species become, therefore, of importance. It must be remembered that the fish used in this study were all very fresh in the market sense of that term. Undoubtedly a greater time between catching and analyzing elapsed in some cases than in others. Additional observations indicate that had all the fish been examined immediately after removal from the water the quantity of this constituent would have been more uniform in the different samples. However, such variations as frequently prevail between the ammoniacal nitrogen in the flesh of the fat-poor and fat-rich tissue cannot be disregarded, nor can they be referred to decomposition changes. A further study of this point is needed. From unpublished data we know that fish packed in ice gradually lose some of their ammoniacal nitrogen owing to the solvent effect of the water. A

decomposed fish which has been standing with ice may show a low content of ammoniacal nitrogen for this reason.

The present report deals with the composition of the edible flesh of the fish exclusively. It is realized that a similar analytical study should be made of the viscera of fishes, especially the livers, before attempting to attach to the present data undue biological significance.

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A METHOD FOR THE PREPARATION OF TAURIN IN LARGE QUANTITIES.*

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For the preparation of large quantities of taurin for experimental work being carried on in this laboratory, we have found that the abalone, *Haliotis*, which can be obtained in quantity on the Pacific coast, is an excellent source for this substance, giving larger yields than can be obtained from ox bile, the more common source for taurin.[1] Kelly[2] found that taurin was present in *Pecten opercularis* and *Mytilus edulis*, and it was likewise found by Mendel[3] in the muscle of *Haliotis*.

Miyake, working in this laboratory with Takeoka,[4] prepared taurin from the abalone. His process was to precipitate the proteins in the aqueous extract obtained by pressing out the juice from the finely ground and boiled abalone muscle, by the addition of potassium aluminum sulfate, filtering, and removing sulfates by barium hydroxide. The excess of the latter was removed by carbon dioxide precipitation and the taurin repeatedly crystallized.

We have used Miyake's method a number of times and found that it was not the most economical and suitable method when large amounts of material are to be used. The difficulties of filtering and washing the enormous precipitate of barium sulfate

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[1] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460. Tauber, S., *Beitr. chem. Physiol. u. Path.*, 1903, iv, 323.

[2] Kelly, A., *Beitr. chem. Physiol. u. Path.*, 1904, v, 377.

[3] Mendel, L. B., *Beitr. chem. Physiol. u. Path.*, 1904, v, 582.

[4] Takeoka, M., *J. Infect. Dis.*, 1917, xx, 442.

led us to look for an easier method. In our hands the method described below has given better yields of taurin than that used by Miyake and has enabled us to work with larger quantities of material at less expense. Several kilos of taurin have been prepared by this method.

After removal of the shell and intestinal tract of the abalone, the muscle is finely ground, heated in a steam sterilizer for a number of hours, and the juice removed by pressure. The resultant cake is reground and again extracted by addition of distilled water and heating. To each 13 liters of the combined extracts is added sufficient glacial acetic acid (about 15 cc.) to make the concentration 0.02 N. This gives the maximum precipitate of protein. After heating for several hours the liquid is transferred to settling bottles, the clear supernatant liquid syphoned off, and the remainder filtered. To the combined filtrates 100 cc. of strong hydrochloric acid are added, the mixture is again heated, and a small additional precipitate removed as before. The clear liquid is evaporated to about one-third of its volume, 500 cc. of strong hydrochloric acid are added, and the mixture boiled until a test sample shows no precipitation of protein on addition of several volumes of alcohol. The liquid is further concentrated and taurin precipitated by the addition of sufficient alcohol to give a concentration of 60 to 70 per cent. On cooling, large amounts of taurin crystals separate, which are filtered and washed with 60 per cent alcohol. The taurin is further purified by boiling with charcoal, crystallizing from water, and washing the crystals with small amounts of water until free from sulfates. Determination of sulfur gave theoretical values. Eight dozen abalones (74 kilos of muscle) gave a yield of 340 gm. of taurin.

An additional yield of 22 gm. was obtained by precipitating the sulfates in the mother liquor by addition of barium hydroxide and crystallizing the taurin. This gave a total yield of 362 gm.

ON THE ELIMINATION OF TAURIN ADMINISTERED TO MAN.*

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Apparently the only experiments concerned with the elimination of taurin administered to man were carried out by Salkowski.[1] He found that taurin taken by mouth was eliminated to the extent of about 87 per cent in the urine, but none of the sulfur appeared in the oxidized form. In fact, the greater portion of the taurin was eliminated as taurocarbamic acid. Symptoms of diarrhea followed the ingestion of 5 gm. doses of taurin. Taurin was found by Salkowski to be toxic for rabbits when given by mouth.

In connection with other experiments being carried out in this laboratory it was desirable to study further the elimination of taurin when administered in various ways to man. For this purpose subjects were placed on simple constant diets and sulfur determinations made in periods preceding and following the administration of taurin. Ringer's solution containing about 6 per cent of taurin was used for the injections. The results obtained are summarized in the tables which follow.

A perusal of the tables shows that the increase of urinary sulfur following the administration of taurin occurs almost entirely in the neutral sulfur fraction, although there is a slight but unmistakable rise in inorganic sulfur following the administration

* Work aided by a grant to the Medical Research Committee from the State Council of Defense, and the George Williams Hooper Foundation for Medical Research.

[1] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460; *Ber. chem. Ges.*, 1872, v, 637.

TABLE I.

Subject 1.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	
1	0.382	0.077	0.071	0.530	
2	0.410	0.084	0.045	0.540	
3	0.424	0.083	0.058	0.566	
Averages.....	0.406	0.081	0.058	0.545	
4	0.483	0.050	1.023	1.557	5 gm. of taurin (S = 1.285 gm.) taken by mouth.
5	0.439	0.062	0.090	0.592	
6	0.430	0.067	0.084	0.581	
7	0.385	0.072	0.079	0.536	

TABLE II.

Subject 2.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	
1	0.473	0.032	0.092	0.597	
2	0.428	0.040	0.101	0.568	
3	0.398	0.053	0.088	0.538	
Averages.....	0.433	0.042	0.093	0.568	
4	0.398	0.042	0.489	0.928	3 gm. of taurin (S = 0.771 gm.) intravenously.
5	0.428	0.029	0.339	0.796	
6	0.437	0.042	0.065	0.544	
7	0.450	0.070	0.061	0.582	

of taurin by mouth, as shown by Subjects 1 and 3, showing that slight oxidation has taken place. The taurin is almost entirely eliminated within the 24 hours following the administration, although a slight rise above the normal is still apparent in the 2 following days. This is especially noticeable in the case of Subject 2. For Subjects 1 and 2 a recovery of 86 and 75 per cent respectively of the taurin sulfur was obtained in the urine. These figures are based on the assumption that the average values obtained in the foreperiod can be taken as a constant for the 3 days following the taurin administration. Owing to a greater varia-

TABLE III.

Subject 3.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	
1	0.448	0.031	0.057	0.544	
2	0.404	0.029	0.099	0.531	
3	0.419	0.042	0.130	0.591	
Averages.....	0.424	0.034	0.095	0.555	
4	0.439	0.035	1.751	2.225	10 gm. of taurin (S = 2.48 gm.) subcutaneously.
5	0.444	0.044	0.132	0.619	
6	0.499	0.053	0.101	0.652	
7	0.706	0.044	1.238	1.988	10 gm. of taurin (S = 2.48 gm.) by mouth.
8	0.516	0.048	0.127	0.692	
9	0.468	0.029	0.119	0.615	
10	0.417	0.030	2.006	2.453	10 gm. of taurin (S = 2.63 gm.) intravenously.
11	0.492	0.031	0.202	0.725	
12	0.560	0.026	0.152	0.739	
13	0.535	0.032	0.109	0.676	

tion in the sulfur content of the diet of Subject 3 the sulfur output for the 3rd day following the taurin administration is taken as the normal value for that period. This gives a percentage recovery of the taurin sulfur in the urine, when given subcutaneously, by mouth, or intravenously, as 62, 59, and 72 per cent respectively. These figures compare favorably with those reported by Salkowski. Pending the development of an easy method for determining the forms of neutral sulfur on which work is under way, we give no results as to the amounts and nature of the compounds by which taurin is excreted in the urine.

It should also be noted that contrary to the findings of Salkowski, no symptoms which could be attributed to the administration of taurin were shown by the human subjects with the doses given, or even when larger amounts were employed. Large amounts may also be given intravenously to rabbits without toxic effect.

SYNTHESIS AND OXIDATION OF TERTIARY HYDROCARBONS.

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The present investigation was evolved from the previous work on the structure of branched chain fatty acids. Levene and Allen[1] have called attention to the lack of a convenient method for determining the location of the tertiary carbon atom in the branched chain fatty acids. An attempt was made to solve the problem by the oxidation of the fatty acids themselves in the hope that the tertiary carbon atom, as the one most susceptible to oxidation, might prove to be the point of disruption of the carbon chain. It is still possible that the solution of the problem may be reached by this mode of attack. Experience has shown, however, that the oxidation of any fatty acid takes place at more than one point of its carbon chain. Because of this the products of oxidation are numerous and it is not always an easy task to formulate the structure of the original molecule on the basis of many fragments. Hence it was thought that an advantage might be gained if prior to oxidation the molecule of the acid could be so transformed as to possess fewer points susceptible to the action of oxidizing agents. It was natural to think in this connection of the hydrocarbons, since every fatty acid is readily convertible into the corresponding hydrocarbon.

Can tertiary hydrocarbons be oxidized by a permanganate solution, or by some other oxidizing agent? The literature on the subject is very meager. In 1901, Zelinsky and Zelikow[2] made the observation that 3-methylpentane is readily oxidized by means of potassium permanganate. Prior to that observa-

[1] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 433.

[2] Zelinsky, N., and Zelikow, J., *Ber. chem. Ges.*, 1901, xxxiv, 2865.

tion it was universally accepted that saturated aliphatic hydrocarbons are not attacked by this reagent.

The observation of the Russian chemists has not been followed up by a study of the products of oxidation, nor has it been extended to observations on other hydrocarbons. The present work was undertaken with the object of obtaining the lacking information.

Since few tertiary hydrocarbons are readily accessible the work naturally fell into two parts, one directed towards the synthesis of tertiary aliphatic hydrocarbons and the other to the study of their behavior towards potassium permanganate. The work is reported in its present incompleteness because one of the authors has accepted a commission with the United States Army.

The most practical and economical way for the preparation of the hydrocarbons was found to be the one based on the reduction of acids obtained by the malonic ester synthesis. In a general way the routine adopted in the work of Levene and Allen was also followed here. However, a marked improvement was introduced in the method of the reduction of esters to the corresponding alcohols. The details are given in the experimental part. Up to the present there were prepared all the intermediate substances leading up to 2-butylhexane as well as the hydrocarbon, also all the intermediate products leading up to 4-butyloctane but not the hydrocarbon.

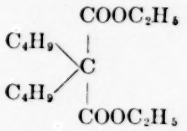
Regarding the behavior of 2-butylhexane towards permanganate it was found that it readily underwent oxidation in an alkaline solution of the reagent. However for the purpose of the study of the products of oxidation, special conditions had to be chosen. Namely, it was found that when the oxidation was permitted to proceed at moderately elevated temperatures (between 80–90°C.), the only oxidation products that could be detected were formic and carbonic acids. On the other hand when the oxidation was conducted at a temperature in the neighborhood of 25°C. evidence was obtained of the formation of butyric acid. This acid was identified as its silver salt. The present experiment was carried out only on a small sample of material. It is intended to continue the work on a larger scale.

The experience gained up to the present is important in as far as it indicates the conditions of experimentation which will per-

mit the isolation of intermediate products of oxidation of the tertiary hydrocarbons.

EXPERIMENTAL.

Diethyl Dibutylmalonate.



This ester was obtained by the action of butyl iodide and sodium ethylate upon diethyl malonate. One molecule of sodium ethylate and one of butyl iodide were added to malonic ester and the mixture was boiled until neutral. After the first substitution was complete another molecule of sodium ethylate and butyl iodide were added and the mixture was again boiled on the water bath until it was no longer alkaline to litmus. The attempt to make both substitutions at the same time, *i.e.* by heating the malonic ester with two molecules of sodium ethylate and of butyl iodide, gave unsatisfactory results. Diethyl dibutylmalonate boils at 153–154° at 14 mm. (corrected).

0.1006 gm. substance gave 0.2464 gm. CO_2 and 0.0938 gm. H_2O.

	Calculated for $C_{15}H_{28}O_4$:	Found:
C	66.58	66.80
H	10.29	10.42

Dibutylmalonic Acid.

This substance was prepared by the saponification of the corresponding ester in the following manner. Malonic ester was converted into diethyl dibutylmalonate as described above. For the preparation of this acid in quantity the ester was not isolated, but to the alcoholic solution of the ester resulting from the treatment of malonic ester with sodium and butyl iodide there was added a solution of potassium hydroxide in the minimal amount of water. The solution was boiled on the water bath under a reflux for 10 hours to complete the saponification, the mixture was then transferred to a large beaker, the alcohol

evaporated, a small amount of water was added, and the acid liberated by the addition of concentrated hydrochloric acid to the cooled solution. The acid separated in solid form. It may be recrystallized from benzene from which solvent it separates in long, transparent, prismatic needles. It is slightly soluble in water but practically insoluble in concentrated salt solution.

114 gm. of this dibasic acid were obtained from 100 gm. of malonic ester, corresponding to a yield of 84 per cent of the theory. Dibutylmalonic acid melts at 163° with slight decomposition.

0.1461 gm. substance gave 0.3260 gm. CO_2 and 0.1199 gm. H_2O.

	Calculated for $C_{11}H_{20}O_4$:	Found:
C	61.15	60.85
H	9.20	9.19

2-Butylhexyli· Acid.

The following will describe a typical experiment by which this acid was prepared. 120 gm. of dibutylmalonic acid were heated in a distilling flask to 180°. This temperature was maintained until the carbon dioxide was no longer evolved. The liquid remaining in the flask was then distilled. At atmospheric pressure 2-butylhexylic acid boils at 255° (corrected); at 16 mm. the boiling point is 153°. The specific gravity at 16° is 0.899. The yield was 90 gm.

0.1681 gm. substance gave 0.4285 gm. CO_2 and 0.1711 gm. H_2O.

	Ca'culated for $C_{10}H_{20}O_2$:	Found:
C	69.76	69.51
H	11.62	11.39

Ethyl 2-Butylhexylate.

130 gm. of the corresponding acid were boiled for 8 hours with four molecules of absolute alcohol and a small amount of sulfuric acid. The yield of ester was 144 gm. Ethyl 2-butylhexylate boils at 114–115° at 15 mm. (corrected).

0.1004 gm. substance gave 0.2652 gm. CO_2 and 0.1060 gm. H_2O.

	Calculated for $C_{12}H_{24}O_2$.	Found:
C	72.00	71.98
H	12.00	11.85

2-Butylhexyl Alcohol.

The method used for the reduction of ethyl 2-butylhexylate to 2-butylhexyl alcohol was essentially the same as that described by Levene and Allen. However, it has been found, in this instance, that the time of reduction can be reduced to half providing the following two changes in procedure are observed: The mixture of alcohol and ester should be added so slowly that no initial cooling is required. That stage of the reaction having been reached when the addition of alcohol ceases to cause more refluxing, the remainder of the alcohol necessary to affect complete solution of the sodium is rapidly added. In this way the reduction may be completed in about 25 minutes and the yield in no way impaired. The yield of alcohol corresponds to between 65 and 70 per cent of that theoretically possible. The acid resulting from saponification was recovered after the manner described by Levene and Allen. The alcohol boils at 218–219° (corrected). Its specific gravity is 0.836.

0.1447 gm. substance gave 0.4044 gm. CO_2 and 0.1802 gm. H_2O.

	Calculated for $C_{10}H_{22}O$:	Found:
C	75.95	76.14
H	13.92	13.93

2-Butylhexyl Iodide.

2-Butylhexyl alcohol was boiled for 5 hours with three molecules of constant boiling hydriodic acid. The yield of iodide was about 80 per cent. The boiling point is 124–125° at 13 mm. (corrected). The specific gravity is 1.267.

0.2242 gm. substance gave 0.1956 gm. AgI.

Calculated for $C_{10}H_{21}I$:	Found:
47.38	47.16

2-Butylhexane.

2-Butylhexane was prepared by the reduction of the corresponding iodide with zinc and glacial acetic acid. 75 gm. of iodide were mixed with 350 cc. of glacial acetic acid and allowed to stand 3 days on the water bath under a reflux. 60 gm. of powdered zinc were added in small amounts. On addition of water two layers were formed; the upper layer of hydrocarbon, after refractionation, weighed 25 gm. The boiling point of 2-butylhexane is 165° (corrected). The specific gravity is 0.738.

0.1010 gm. substance gave 0.3726 gm. CO_2 and 0.1394 gm. H_2O.

	Calculated for $C_{16}H_{32}$:	Found:
C	84.51	84.41
H	15.49	15.41

Diethyl 2-Butylhexylmalonate.

90 gm. of malonic ester (an excess of 20 per cent above the theory) were converted into the monosodium derivative by the addition of 10.6 gm. of sodium in alcohol; 125 gm. of 2-butylhexyl iodide were slowly added and the mixture was boiled on the water bath until neutral. The yield of the substituted ester was 90 gm. This ester boils at 180° at 14 mm. (corrected).

0.1064 gm. substance gave 0.2664 gm. CO_2 and 0.0972 gm. H_2O.

	Calculated for $C_{17}H_{32}O_4$:	Found:
C	68.00	68.29
H	10.60	10.23

2-Butylhexylmalonic Acid.

This acid was prepared from the corresponding ester by saponification with potassium hydroxide in alcohol solution. It crystallizes from low boiling petroleum ether in transparent, rhombic needles which melt at 88° (corrected).

0.1018 gm. substance gave 0.2384 gm. CO_2 and 0.0876 gm. H_2O.

	Calculated for $C_{13}H_{24}O_4$:	Found:
C	63.93	63.87
H	9.83	9.63

4-Butyloctylic Acid.

The method used for the preparation of this acid was exactly analogous to that described for the preparation of 2-butylhexylic acid. The boiling point is 173–174° at 12 mm. (corrected). The specific gravity is 0.901.

0.1034 gm. substance gave 0.2736 gm. CO_2 and 0.1108 gm. H_2O.

	Calculated for $C_{12}H_{24}O_2$:	Found:
C	72.00	72.16
H	12.00	12.00

Ethyl 4-Butyloctylate.

In the preparation of this substance 36 gm. of 4-butyloctylic acid were boiled for 6 hours with four molecules of absolute alcohol and a few drops of sulfuric acid. The refractionated product weighed 32 gm. It boils at 139° at 10 mm. (corrected).

0.1000 gm. substance gave 0.2692 gm. CO_2 and 0.1104 gm. H_2O.

	Calculated for $C_{14}H_{28}O_2$:	Found:
C	73.67	73.41
H	12.24	12.35

4-Butyloctyl Alcohol.

28 gm. of the above ester upon reduction yielded 14 gm. of alcohol. The boiling point is 139° at 15 mm. Its specific gravity is 0.841.

0.1266 gm. substance gave 0.3584 gm. CO_2 and 0.1266 gm. H_2O.

	Calculated for $C_{12}H_{26}O$:	Found:
C	77.29	77.20
H	13.57	13.90

4-Butyloctyl Iodide.

This iodide was prepared by boiling a mixture of 10 gm. of the corresponding alcohol with four molecules of constant boiling hydriodic acid. The yield of iodide was 14 gm. It boils at 143° at 8 mm. Specific gravity, 1.194.

0.1992 gm. substance gave 0.1566 gm. AgI.

	Calculated for $C_{12}H_{25}I$:	Found
I	42.90	42.32

Oxidation of 2-Butylhexane.

The hydrocarbon was oxidized by allowing it to stand in contact with an alkaline solution of potassium permanganate at room temperature for about 4 weeks. Three equivalents for permanganate were used, enough water being added to make the concentration of the permanganate 5 per cent at the beginning of the oxidation. Only a small amount of the hydrocarbon was oxidized. The unoxidized portion was separated from the aqueous layer, the latter acidified and distilled with steam into diluted ammonia. This distillate was concentrated to a small volume *in vacuo* and the fatty acid precipitated as the silver salt.

0.0971 gm. silver salt gave on ignition 0.0531 gm. silver.

	Calculated for $C_4H_7O_2Ag$:	Found:
Ag	55.38	54.68

COPPER-PHOSPHATE MIXTURES AS SUGAR REAGENTS.

A QUALITATIVE TEST AND A QUANTITATIVE TITRATION METHOD FOR SUGAR IN URINE.

By OTTO FOLIN and W. S. McELLROY.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, January 25, 1918.)

In connection with a previous investigation[1] it was observed that alkaline phosphates can hold copper hydroxide in solution. So far as we know phosphates have never been used as a solvent for copper hydroxide in the preparation of reagents for sugar, yet it was thought that phosphates might possess distinct advantages for these purposes. They are less expensive than tartrates, citrates, or glycerol, they do not reduce sugar, and they tend to regulate the degree of alkalinity at a lower level of hydroxyl ion concentration than is obtained by carbonates alone; a given amount of sugar should consequently reduce more copper in such solutions.

The serviceability of phosphates in the preparation of copper reagents for sugar was soon demonstrated, but they can be used in so many different ways that it has been rather difficult to decide which combination to adopt. (Potassium salts have been excluded on account of their cost.)

Qualitative Test for Sugar in Urine.—One good copper-phosphate reagent is made as follows: Dissolve 100 gm. of sodium pyrophosphate (U. S. P.), 30 gm. of crystallized disodium phosphate, and 50 gm. of anhydrous sodium carbonate, in about 1 liter of water. To this solution add 13 gm. of copper sulfate previously dissolved in about 200 cc. of water. The solution seems to keep indefinitely, except that in cold weather some phosphate may crystallize out. The reagent should therefore not be left in very cool places, although it makes little or no difference whether a small part of

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 477.

the phosphate has formed a crystalline cake in the bottom of the container.

The solution is used exactly as is Benedict's reagent for sugar: To about 5 cc. in a test-tube add 5 to 8 drops of urine (not over 0.5 cc.) and boil 1 minute, or heat in boiling water for 3 to 5 minutes. Minute traces of sugar are indicated by various grades of turbidity, as in Benedict's test; larger amounts, by unmistakable cuprous oxide precipitates. The test is quite as sensitive and reliable as Benedict's and a trifle more prompt. The only point of distinction which should be noted in the use of this reagent is that unless a very marked turbidity is noted in the hot solutions the result must be regarded as clinically negative. The slight turbidity occurring after cooling represents only the reducing action of normal urine.

Quantitative Titration of Sugar in Urine.—The quantitative method described below for the titration of sugar in urine we believe to be practical and inexpensive.

The only solution required is an acidified copper sulfate solution containing 60 gm. of $CuSO_4.5HO_2$ per liter.[2] 5 cc. of this solution correspond to 25 mg. of dextrose or levulose,[3] 45 mg. of anhydrous maltose, or 40.4 mg. of anhydrous lactose. The other necessary reagent is a dry mixture containing 100 gm. of disodium phosphate crystals ($HNa_2PO_4.12H_2O$), 60 gm. of dry sodium carbonate ($Na_2CO_3.H_2O$), and 30 of sodium or potassium sulfocyanate. These salts are mixed together in a large mortar;

[2] Pure copper sulfate solutions gradually decompose slightly giving a sediment of copper hydroxide, or silicate. This decomposition is presumably caused by the solvent action on the glass. To prevent it add about 2 cc. of concentrated sulfuric acid for each 30 gm. of copper sulfate used, in making the standard 6 per cent copper sulfate solution.

[3] In titrations of levulose the oxidation is so rapid and complete that practically no attention need be paid to the time factor, so important in the titration of other sugars. A levulose titration can be finished in about 2 minutes.

The dextrose and levulose used were Kahlbaum preparations giving correct polariscope values. The milk sugar was purified by recrystallization from dilute alcohol and acetone. The maltose was a sample of Kahlbaum's, but contained traces of dextrin. It was purified by dialyzing for 3 hours, and the maltose value determined by the polariscope in the dialysate was accepted as representing pure maltose. We have not been able to obtain any strictly pure maltose.

the mixture keeps indefinitely.[4] Less than 1 gm. of the sulfocyanide, now so difficult to obtain, is consumed for each titration.

The titrations are made in test-tubes. By this innovation the following advantages are gained: (1) the cost of chemicals is greatly reduced; (2) the preliminary heating period is very short; (3) there is no need for regulating the flame to some definite speed of boiling as this is accomplished by moving the test-tube sidewise through the flame; (4) the disappearance of the last traces of blue color is more sharply marked because the volume of liquid is small; (5) there is little or no return of any blue or green color at the end of the titration. Independently of the shape of vessel used for the titration the return of a blue or greenish color by reoxidation is very slow in our titration mixtures. Using pure sugar solutions and test-tubes the titrated solutions remain colorless for several hours.

The titrations are made on undiluted urine even when as much as 6 or 7 per cent of sugar is present. This simplification is made possible by attaching to the tip of an ordinary 25 cc. glass-stoppered burette another tip, consisting of a glass tube drawn out at one end to an almost capillary bore. It is a very simple matter, and requires only a few minutes' work, to make a dozen such tips which will deliver between 45 and 55 drops of urine per cc. We have found these fine tips very helpful in many other kinds of accurate titrations; they permit the measurement of a fraction of 1 cc. with almost as great an accuracy as the measurement of 5 cc. with an ordinary burette.

The burettes carrying accessory tips are most conveniently filled by suction instead of by pouring in the urine from the top. This mode of filling the burette avoids all spilling of urine, all foaming within the burette, and all waiting for the meniscus to reach the proper level. The filling of the burette by suction has also the advantage that it eliminates the necessity of rinsing the burette with the sugar solution or urine to be titrated. A 5 per cent urine can be used directly after a 0.5 per cent one, or *vice versa*. That this is the case can be shown by filling the burette with water after it has just been emptied from a urine containing

[4] When first made the salt mixture tends to cake or set a trifle. If it is left in the mortar or other vessel, covered with paper, over night and is then stirred up once more, before bottling, it does not harden again.

5 per cent of sugar and then testing the water in the lower part of the burette for sugar. It contains no sugar. For the suction we use a rubber tube about 30 cm. long, having a glass tip at the lower end; we attach it to the burette by means of a small rubber stopper and a bent glass tube; since the burette needs no cleaning or rinsing the suction arrangements need seldom be removed. The rubber tube can of course be omitted. The essential point is merely to fill the burette, like a pipette, from the bottom by means of suction.

Sugar Titration.

Fill the burette as described (by suction) to the zero mark. Into a test-tube introduce a pebble, 5 cc. of the copper sulfate solution (corresponding to 25 mg. of dextrose), and 4 to 5 gm. of the dry salt mixture. Shake and heat until a clear solution is obtained; this takes usually only a few seconds. Add 25 drops of the urine and boil *very gently* for 2 minutes. If all the copper has been reduced the urine contains probably more than 5 per cent sugar. In that case the determination must be started over again. If the copper is but slightly reduced, yielding only a small amount of white precipitate, add more urine, from 10 to 25 drops depending on the amount of unreduced remaining copper, and boil *gently* for another minute. If at the end of the second boiling period most of the copper has been reduced, complete the titration by the drop system, keeping count of the number of drops added and boiling 1 minute after each subsequent addition of urine. At the end of the titration determine how many additional drops of urine are required to give a volume of 1 or 2 cc. For example, if 37 drops were used in reducing the copper another 15 drops may be required to give a volume of 1 cc. and the volume of urine taken is therefore $\frac{3}{5}\frac{7}{2}$ of 1 cc. Or if 70 drops were used and 36 more are needed to give a volume of 2 cc., then the volume of urine used is $\frac{7}{5}\frac{0}{3}$ of 1 cc. If 50 drops of urine have not reduced most of the copper, then the titration is finished by the addition of larger amounts of urine, without counting the drops, and at the end of the titration the volume of urine taken is determined, as in any other titration, by the reading on the burette. 2.5 divided by the volume of urine

taken, whether this is several cc. or a fraction of 1 cc., gives the per cent of sugar in the urine. It is, of course, desirable to confirm the results of the titration by a repetition. In this case the first addition of urine should be only 2 or 3 drops less than the full amount of urine required and the first boiling period should be 3 minutes. The titration is then finished by boiling 1 minute after each subsequent addition of urine. The total boiling period must not be less than 4 minutes, and if it is more than 7 the results obtained tend to be a trifle high. 5 to 6 minutes is the time for which the copper value has been adjusted.

For accurate work two precautions must be observed in connection with the drop system of sugar titration. The drops should be delivered at a slow rate of speed, not faster than 1 drop per second. And the portion of the burette most used, the first 2 cc., should be calibrated. (1 cc. of distilled water weighs about 997 mg.) After the first 2 cc. of the burette have been calibrated by weight the remaining parts of the burette can be calibrated in a few minutes by means of the drop system.

Special Burette for Sugar Titrations.—While very satisfactory results are obtained in the titration of undiluted sugar urines by means of ordinary burettes and the drop system described in the preceding section, we recognize that many who do or teach sugar titrations will prefer to pay for fine burettes and thus do away with the necessity of keeping count of the number of drops used in each titration. Further, a small burette has the advantage that two or more titrations can be made with as little as 10 cc. of urine or sugar solution—an important consideration when teaching large classes, and also for certain kinds of work, as, for example, for the titration of sugar in human milk. We recommend for this work 5 cc. burettes with glass stop-cocks graduated in 0.02 of a cc.[5] These burettes can be read to within 0.01 of 1 cc.

These burettes must be filled by suction. The tips of the burettes are necessarily too coarse to deliver more than 25 or 30 drops per cc. The fine accessory tips delivering 45 to 55 drops per cc. already described are therefore highly desirable in connection with these burettes. In other words, the burettes should be used just as ordinary burettes are used in the drop system

[5] Our burettes are made by the Meyer Camera and Instrument Company, 31–33 East 27th Street, New York.

described above except that the counting of the drops is omitted.

The sugar titration described above has been adapted to the use of test-tubes instead of evaporating dishes, beakers, or flasks, and it has been adapted to the use of undiluted urine. The objection to the traditional dilution of urine to a sugar concentration of 0.5 to 1 per cent is that it cannot be properly made except on the basis of a preliminary sugar titration. (In the hands of medical students with a limited supply of measuring utensils the most frequent cause of gross errors in sugar determinations is one or another error or carelessness in connection with the dilution.) Our sugar titration can, of course, also be made with sugar solutions containing only one-half of 1 per cent of sugar and can therefore be made with sugar urines diluted to such a sugar concentration. It can also be adapted to the use of flasks instead of test-tubes, but only at the expense of chemicals. For those who prefer to use flasks, we recommend that the standard copper solution contain 3 per cent instead of 6 per cent copper sulfate. For 10 cc. of this solution (corresponding to 25 mg. of dextrose) 10 gm. of the dry alkaline salt mixture should be taken. If much less salt than that prescribed is used the white cuprous sulfocyanide is hydrolyzed and traces of red cuprous oxide begin to appear and the end-point of the titration becomes less readily visible. We believe that very few who have once become familiar with the use of test-tubes in sugar titrations will go back to the use of flasks, beakers, or other vessels.

Sugar in Urine.

No. of urine.	New method.	Benedict's method.	By the polariscope.
	per cent	*per cent*	*per cent*
1	1.8	1.78	1.81
2	4.16	4.2	4.12
3	2.94	2.94	2.99
4	1.41	1.43	1.39
5	0.98	1.05	0.98
6	6.66	6.56	6.63
7	1.56	1.54	1.52
8	2.84	2.96	2.89
9	4.5	4.56	4.45
10	3.62	3.74	3.68

Sugar urines frequently contain albumin. Albumin does not destroy the accuracy of the sugar titration described in this paper. Because of the foaming produced by albumin it is, however, desirable, indeed necessary, to use rather large test-tubes and to boil cautiously so that the contents do not boil over. The albumin does alter the appearance and probably also the composition of the cuprous precipitate, making it more flocculent and bulky; but, as already stated, the end-point of the titration is not changed or obscured by the presence of albumin.

THE DETERMINATION OF LACTOSE IN MILK.

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(Received for publication, January 25, 1918.)

In this paper we describe two methods for the determination of the sugar in milk—a titration method and a colorimetric method. Of the two procedures we believe the titration to be the more accurate. The colorimetric method has, however, the advantage that by means of it a very large number of determinations can be made more or less simultaneously.

I. Titration Method.

In the sugar titration method of Folin and McEllroy 40.4 mg. of anhydrous lactose have the same reducing value as 25 mg. of dextrose. The lactose value was determined by us because it was thought that the method might prove quite as serviceable for the titration of the lactose of milk as for the titration of the dextrose of urine. Since albumin does not interfere with that titration it was hoped that the lactose of milk could be titrated directly, that is without any preliminary preparation of protein-free filtrates. A remarkably simple method for the determination of the sugar in milk would thus be secured. Our conjecture turned out to be correct, for we have encountered no difficulties in titrating the lactose of milk without first removing the protein and the fat. By means of large numbers of check determinations, using protein-free filtrates and determining the sugar by titration, by the polariscope, as well as colorimetrically, we have satisfied ourselves that the preliminary removal of the protein materials is superfluous. Omitting the preparation of protein-free milk filtrates eliminates not only the most tedious feature of the older methods for titrating the sugar in milk, but it also does away

with the cumbersome "corrections" for the volume occupied by the protein-fat precipitates.

Because of the extreme variations in the sugar content of urine it seemed desirable to be able to titrate it without preliminary dilution. In the case of milk the circumstances are entirely different, for the variations in the sugar content of this secretion are relatively very small, and a *suitable* preliminary dilution, without any uncertainty and without any approximate preliminary sugar titration, can be made. It has therefore seemed to us rather better to dilute milk before titrating, though the titration can be made quite well without any such dilution. We recommend a dilution of 1:4 (25 cc. in a 100 cc. flask) for cow's milk and 1:5 (5 cc. in a 25 cc. flask) for mother's milk. In emergencies, as when the quantity of human milk available for the sugar titration is very small, a dilution of 1:6 (2 cc. added to 10 cc. of water) can be used.

The titration is made as follows: Into a large test-tube introduce 2.8 to 3.4 cc. of the diluted milk (that is, nearly the full amount expected), 5 cc. of a 6 per cent copper sulfate solution, a pebble (to prevent bumping), and 4 to 5 gm. of a dry salt mixture (made by mixing, in powdered form, 100 gm. of disodium phosphate, 60 gm. of anhydrous sodium carbonate, and 30 gm. of sodium or potassium sulfocyanate). Shake, and boil gently for 4 minutes before adding any more milk. At the end of this time add more milk (0.02 cc. to 0.10 cc., depending on the amount of blue color remaining) and boil again. After each addition of milk (except the first) boil 1 minute. The total boiling period should be 5 to 7 minutes.

For the measurement of the milk we recommend the 5 cc. burette and other accessories, mentioned in the preceding paper in connection with the titration of sugar urines.[1]

Calculation: 4.04 multiplied by the degree of dilution (4, 5, or 6) and divided by the titration figure, gives the per cent of lactose present.

[1] We have up to date made over 200 sugar determinations by this new titration method—most of them on human milk. This work has developed into a relatively extensive study of the sugar content of human milk and will be published later.

II. Colorimetric Method for the Determination of Lactose.

Dehn and Hartman[2] have already described a colorimetric picrate method for the determination of the sugar in milk. Our procedure is materially simpler, and it gives, we believe, as accurate results as it is possible to obtain by a colorimetric picrate method.

The method is as follows: With an accurate 2 cc. pipette transfer 2 cc. of milk to a 100 cc. volumetric flask previously half filled with saturated picric acid solution. Fill to the mark with saturated picric acid solution, shake, and filter. Transfer 5 cc. of the filtrate to a large test-tube or 100 cc. volumetric flask, add 15 cc. of saturated picric acid solution and 3 cc. of 20 per cent sodium carbonate solution. Mix and place in a boiling water bath for 15 minutes. Cool and dilute to a volume of 100 cc. The standard consists of a solution of lactose in saturated picric acid solution. For use with cow's milk this solution should contain 5 mg. of lactose in 20 cc. For use with human milk it is necessary to employ two standards; namely, one containing 5 and the other containing 7 mg. of lactose in 20 cc. To 20 cc. of the standard add 3 cc. of the sodium carbonate solution and heat simultaneously with the milk filtrate. Cool, dilute to 100 cc., and compare the colors in the usual manner in a suitable colorimeter.

According to our experience accurate results cannot be uniformly obtained except by heating the standard simultaneously with the unknown and it is therefore absolutely necessary to prepare a new standard for each set of determinations. Nor can the color values of the standard and the unknown be more than 20 per cent apart.

In the following table are given a few figures representing parallel determinations of lactose in milk made by the colorimetric and by the titration methods.

[2] Dehn, W., and Hartman, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 404.

Determinations of Lactose in Cow's and in Women's Milk.

No.	Lactose.	
	Colorimetric method.	Titration method.
	per cent	*per cent*
Cow 57	4.42	4.41
" 41	4.08	4.30
" 37	4.13	4.22
" 22	4.54	4.46
" 39	4.87	4.84
" 45	4.51	4.54
" 31	4.21	4.23
" 31	4.37	4.45
" 44	4.60	4.50
" 38	4.64	4.68
Mother 1	3.08	3.10
" 8	5.73	5.75
" 9	5.07	5.05
" 10	4.79	4.83
" 12	6.73	6.63
" 13	6.64	6.53
" 16	5.87	5.87
" 17	6.37	6.40

NITROGEN CONTENT OF BACTERIAL CELLS.

I. METHOD.

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The production of acids, gases, and intermediate products, of toxins, agglutinins, and many other protein complexes serves to differentiate strains and groups of organisms and at the same time furnishes us with considerable interesting data in regard to bacterial metabolism. On the other hand, we have very little information as to the proximate and ultimate composition of bacteria. The difficulties attending the growth of the organism in sufficient quantity for accurate analysis and the difficulties of the analytical technique have largely prevented the development of this aspect of the work.

We know that higher plants differ from each other in their nitrogen content, and in the compounds characteristic of each. Enough work has already been done to indicate that wide differences may exist in the nitrogen content of bacteria. Nicolle and Alilaire (1) and Wheeler (2) give the results tabulated below for the nitrogen content of various organisms.

The results show the wide variation in the nitrogen content of various organisms and also the great discrepancies between analyses of the same organism by different persons. These results are of doubtful value, probably because of the variety of conditions maintained during growth, and because the technique allows errors of considerable magnitude where such small amounts of material were to be used. It was decided to apply the Folin method (3) to our problem and after certain adaptations we arrived at the following method for determining the nitrogen content of bacteria.

Kind of organism.	Nitrogen.	
	Nicolle and Alibaire.	Wheeler.
	per cent	*per cent*
B. proteus vulgaris	10.73	6.791
Typhoid	8.28	11.55
Anthrax	9.22	10.28
Colon	10.32	10.655
B. pyocyaneus	9.79	10.843
" *subtilis*	—	5.964
" *violaceus*	—	11.765
" *tuberculosis* "air-dried"	—	9.27
Chlorella vulgaris	3.96	—
B. dysenteriæ liquefaciens	8.89	—
" *pneumoniæ*	8.33	—
" *psittacosis*	9.55	—
Glanders	10.47	—
Sarcina aurantiaca	—	11.46

Method.

A battery of ten hard glass test-tubes was set up together with a sand bath and Bunsen burners to furnish the required temperature. Inverted funnels with bent stems connected with rubber tubes to a suction bottle were placed over the tubes to remove the fumes and exclude the dust.

The bacteria used in the experiment were cultures of *Bacillus diphtheriæ* which had been isolated from the throats of persons suffering from the disease. These bacteria were grown on Loeffler's blood serum medium slants for 72 hours. It usually required eight of these tubes to furnish enough material for one determination. The growth of organisms was removed without disturbing the solid medium, by a glass spade with rounded edges, made by flattening a small glass rod. The material was spread on a tared cover-slip and dried in a calcium chloride desiccator at 37°C. for 72 hours.

On account of the small amount of the material obtainable from a reasonable number of tubes, the weighing was done on an assay balance which was sensitive to 0.000005 gm. The weighing glasses were the thin, 15 mm. square cover-slips used in bacteriological work. They were cleaned in concentrated

nitric acid, washed in distilled water several times, and then finally in alcohol, out of which they were wiped dry when used.

Into each of the ten tubes was placed the following charge:

	gm.
Potassium sulfate	2.0
Copper sulfate	0.2
Sulfuric acid, concentrated	5.0

Weighed cover-slip and material.

The digestion was continued for 15 minutes after the liquid had become colorless. In practically all the material digested, there was noticed a small quantity of material which digested with difficulty. After the digest had cooled somewhat it was diluted with water, made alkaline with sodium hydroxide, and

TABLE I.

Cover-glass and dry bacteria.	Dry bacteria.	Net weight of nitrogen.	Nitrogen.
gm.	*gm.*	*gm.*	*per cent*
0.080280	0.004655	0.000370	7.9
0.081060	0.004670	0.000390	8.3
0.076380	0.005730	0.000485	8.5
0.076925	0.007095	0.000575	8.1
0.085820	0.003360	0.000290	8.6
0.070600	0.005420	0.000440	8.2
0.085050	0.007300	0.000581	8.0
0.058450	0.004040	0.000357	8.8
0.056470	0.002880	0.000245	8.9
0.079020	0.004810	0.000413	8.7
0.073330	0.001485	0.000120	8.4
0.080460	0.002575	0.000250	9.7*
0.097390	0.003390	0.000291	8.6
0.089075	0.001350	0.000154	11.4*
0.065480	0.006170	0.000470	7.6
0.068180	0.004625	0.000490	10.5*

* On account of the great variance from the average it seems permissible to omit these results from the average.

aspirated with ammonia-free air into 0.1 N sulfuric acid. The acid solution containing the ammonium sulfate was Nesslerized and compared with a standard solution of ammonium sulfate similarly Nesslerized, by means of the Duboscq colorimeter. A convenient strength of standard to use, if the quantity digested is about 5 mg., contains an ammonia equivalent of 0.0000025 gm. of nitrogen per cc. With every set of determinations two blanks were run and the average of these was subtracted from the nitrogen found.

The results of sixteen separate determinations are given in Table I.

A nitrogen determination was made on the Hoffmann bacillus, a diphtheroid organism, by this method with the results as recorded in Table II.

TABLE II.

Nitrogen Content of Bacillus hoffmanni.

Cover-glass and dry bacteria.	Dry bacteria.	Net weight of nitrogen.	Nitrogen.
gm.	*gm.*	*gm.*	*per cent*
0.074110	0.002900	0.000277	9.6
0.075590	0.003730	0.000356	9.5
0.100210	0.006235	0.000600	9.6
0.071810	0.009890	0.001020	10.3

In order to determine the accuracy of the method, a macro-Kjeldahl determination was made on a larger quantity of the dried diphtheria bacteria with the following results:

No. 1..... 7.9 per cent nitrogen.
" 2..... 8.1 " " "

SUMMARY.

In summing up the results of these determinations the following points seem important:

1. The nitrogen content of *Bacillus diphtheriæ* is

	per cent
Regular Kjeldahl method	8.0
Micro-Kjeldahl method	8.35

The nitrogen content of *Bacillus hoffmanni* is found to be 9.75 per cent by this method. Under like cultural conditions then, these two organisms show marked differences in this particular.

2. The sources of error met when an analysis is made are overcome as follows: (*a*) The possibility of contamination of the material with small pieces of medium is eliminated by the use of a molded glass spade which rubs off the growth without scratching the medium. (*b*) The presence of moisture in the organism when weighed. The organisms were dried under like conditions which were purely arbitrary but which were found to be adequate for reducing the film to constant weight. The analytical results themselves are sufficiently alike to show that the moisture content must have been uniform for each type. (*c*) The error in weighing. That this error is only apparent is seen by comparing the accuracy of the scale used with the amount weighed. (*d*) The error in ammonia obtained from reagents. This was entirely accounted for by running measured blanks with every determination. (*e*) Loss of ammonia by aspiration. In the first few practice runs there was experienced a noticeable error at this point; however, the rate of aspiration at the start was reduced and the total length of time prolonged which overcame this source of error.

CONCLUSIONS.

It is possible to determine the nitrogen content of any bacterium which will grow on a solid medium without liquefaction of that medium, by this method, provided as much material as 5 mg. can be obtained.

It is our intention to study further the nitrogen differences in bacteria of different groups and of different members of the same group.

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3. Folin, O., and Farmer, C. J., A new method for the determination of total nitrogen in urine, *J. Biol. Chem.*, 1912, xi, 493.

IONIZATION OF PROTEINS AND ANTAGONISTIC SALT ACTION.

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I.

In 1899 Wolfgang Pauli[1] and the writer[2] independently reached the conclusion that electrolytes when acting on proteins formed ion-protein compounds. The writer anticipated that these ion-proteins would explain the mystery of many life phenomena. He was especially interested in one of the most universal physiological actions of salts; namely, the antagonistic salt action, for which the annihilation of the effects of a high concentration of a salt with univalent cation, *e.g.* NaCl, by a low concentration of a salt with bivalent cation, *e.g.* $CaCl_2$, is perhaps the best known example. Although he and many others tried to demonstrate this type of antagonism in proteins they never succeeded. It was a further disappointing fact that Hardy[3] found that globulins apparently form electrically neutral compounds with neutral salts and this seemed to harmonize with the older observations of Liebermann and Bugarszky. Pauli[4] had expressed the idea that a low concentration of salts ionizes globulins and thereby causes their solution, but even he assumed not a real chemical combination but adsorption between the globulin and the salt.

Meanwhile, many workers, and especially Pauli and his pupils, had developed a number of methods for discriminating between the chemical behavior of ionized and non-ionized proteins, but

[1] Pauli, W., *Arch. ges. Physiol.*, 1899, lxxviii, 314.

[2] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303; *Am. J. Physiol.*, 1900, iii, 327.

[3] Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 251.

[4] Pauli, *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

the surest method of producing ionized proteins was to treat proteins with either acid or base. It was generally found that when a protein with little tendency to electrolytic dissociation is treated with acid of the right concentration a protein salt is formed which undergoes a strong electrolytic dissociation, while the addition of a neutral salt diminishes this degree of dissociation, as was to be expected. Loeb and Wasteneys,[5] and later the writer alone,[6] were able to show that the toxic effect of acid can be diminished or suppressed by the addition of neutral salts. This was the first direct support of the idea that antagonistic salt action was due to a transformation of ionized protein into electrically neutral protein.

The second case where it seemed possible to attribute antagonistic salt action to a transformation of ionized into non-ionized protein consisted in the observation that the diffusion of a low concentration of KCl into the egg of *Fundulus* is impossible or extremely slow when this salt is alone in solution, but is rendered possible through the addition of a small amount of a second salt, *e.g.* NaCl ("salt effect"), and that the diffusion of KCl is rendered impossible again when more of the second salt is added. The analogy with the solubility of globulin suggested that the addition of a small quantity of the second salt caused an ionization of some protein in the membrane of the egg which was suppressed again by the addition of more salt; and the writer expressed this idea in a preliminary notice.[7] But this suggestion faces the uncomfortable fact already referred to that according to Hardy globulins form non-ionized molecules with neutral salts.[3]

The writer, however, has found a method which suggests strongly that the antagonism between NaCl and $CaCl_2$ is due—at least in the case to be discussed—to the fact that an ionization of protein is caused by NaCl and that this ionization or its effect upon the swelling is suppressed by a comparatively small quantity of $CaCl_2$.

[5] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, **xxxix**, 167.

[6] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii, 147.

[7] Loeb, *Proc. Nat. Acad. Sc.*, 1916, ii, 511.

Some of the experiments have already been published.[8] Into a cylindrical funnel 2 gm. of finely powdered *non-bleached*[9] Cooper's gelatin are put; the powder is held in the cylinder by a circular piece of filter paper. Three such funnels each with 2 gm. of gelatin are prepared. The one (I) is perfused six times in succession with 25 cc. of distilled water; the second (II) is perfused twice with 25 cc. M/8 NaCl and then four times with 25 cc. of distilled water; the third (III) is perfused six times with 25 cc. M/8 NaCl. In I and III a moderate swelling occurs, which soon reaches its maximum. The gelatin in II, first treated with NaCl and subsequently with distilled water, swells several hundred per cent more than either the gelatin treated only with distilled water (I) or the gelatin (III) treated only with NaCl.

The explanation of this experiment is as follows. Gelatin II forms under the influence of a comparatively high concentration of NaCl (M/8 or M/4) a compound with NaCl which is capable of ionization. This ionization is lowered through the presence of the highly concentrated NaCl solution. When, however, this latter solution is washed away by successive perfusions of the gelatin with distilled water the gelatin-NaCl compound can dissociate into gelatin and an inorganic ion, the nature of which we shall discuss later. It has been shown by Pauli and by Procter[10] that the swelling of protein under the influence of acid or base is due to the ionization of protein by the acid and the writer assumes that the increase in the amount in swelling of the mass of gelatin first treated with NaCl and then washed with distilled water is due to the fact that part of the gelatin is transformed into protein ions by the salt, and that this ionization and the swelling can only appear when the NaCl solution held in the capillary spaces of the powder is washed away. This statement is supported by the following facts.

1. A large number of funnels are prepared, each with 2 gm. of powdered gelatin and each is perfused twice with 25 cc. M/8 NaCl. Subsequently each funnel is perfused three times with

[8] Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

[9] Bleached gelatin as well as bleached pig's bladder does not give the same results, probably on account of an alteration in the constitution of the protein.

[10] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

25 cc. of a definite NaCl solution lower than M/8 NaCl; namely, M/16, M/32, M/64, M/128, etc., down to M/2048 or lower. It is found that after three perfusions the additional swelling of the gelatin becomes noticeable in the funnel which has been washed with M/64 or less concentrated NaCl solution and that the additional swelling increases rapidly with the diminution of the concentration of the NaCl solution. The explanation is that concentrations of NaCl above M/64 suppress the electrolytic dissociation of the gelatin-NaCl compound to such an extent that the swelling will not exceed that caused when the gelatin is perfused permanently with M/8 NaCl. Concentrations of M/64 and below no longer suppress the ionization of the gelatin salt completely and the suppression is the smaller the greater the dilution of the NaCl used for perfusion. Hence the swelling of the gelatin increases rapidly with the dilution of the NaCl solution below M/64 and reaches a maximum when distilled water is used for the perfusion. Table II gives adequate examples. This fact had already been published.[8]

2. The second proof consists of the fact that when the gelatin powder is first perfused with M/8 NaCl and then with a solution of a non-electrolyte, the increasing dilution of the non-electrolyte is without effect. Table I illustrates this statement. The table gives the result of four sets of experiments. In each set about fourteen cylindrical funnels contained 2 gm. of powdered gelatin and each funnel was perfused twice with 25 cc. M/8 NaCl. After this the various funnels of one series were perfused with solutions of either NaCl or glycerol or cane sugar or ethyl alcohol of increasing dilutions. The swelling was measured after three perfusions. In order to allow all the liquid not held by the gelatin (or by adhesion) to filter out the final measurement was taken after 24 hours. Precautions were taken to avoid error by evaporation. Since the cross-section of all the cylindrical funnels was the same the increase in the height of the cylindrical mass of gelatin above the height of the mass reached by the perfusion with M/8 NaCl may serve as a measure of the additional swelling.

The difference between the influence of the electrolyte and the non-electrolyte is very striking. Gelatin treated with M/8 NaCl does not show any further swelling when treated with M/8 or M/16 NaCl; from M/64 on the swelling begins, gradually increas-

ing and reaching its maximum at M/1024. Gelatin treated with M/8 NaCl and then treated with solutions of non-electrolytes from 2 M down swells to the same extent as if it were treated with distilled water. This harmonizes with the assumption that the swelling is determined by the degree of electrolytic dissociation of the gelatin.

3. It has been shown by Pauli and his fellow workers that ionized protein can no longer be precipitated by alcohol.[4] If the additional swelling of gelatin in distilled water caused by the previous treatment with M/8 NaCl was due to an increase in ionization such gelatin should resist precipitation by alcohol. This is most strikingly the case. When we dissolve the three kinds of

TABLE I.

	Increase in swelling of powdered gelatin (which had first been perfused with 50 cc. M/8 NaCl) after three perfusions with 25 cc. of													
	4 M.	2 M	M	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	H_2O
NaCl.....						0	0	2.5	6	15	21	33.5	42	
Glycerol.....	34	37	40	40	40	43	42	42	40	41	41	41	43	42
Cane sugar.....		35	41	49	49	51	51	50	50	51	51	51		48
Ethyl alcohol..	42.5	48.5	54	48.5	48.5	48.5	49.5	49.5	49.5	47	47.5	47		50.5

powdered gelatin, I treated with water alone, II treated first with M/8 NaCl and then with water, and III treated with salt (M/8 NaCl) alone, and measure the quantity of alcohol needed to precipitate 5 cc. of a 3 per cent gelatin solution of each of the three samples, we shall find that it requires about 5 cc. of 95 per cent alcohol to cause a heavy precipitate in I, and about 7 cc. to cause the same precipitate in III; while in II no precipitate is formed even by the addition of 20 cc. or more of alcohol; the solution assumes only a bluish tint upon the addition of about 20 cc. of alcohol.

4. It was shown in the previous paper that any neutral salt with univalent cation acts like NaCl upon powdered gelatin, inasmuch as a perfusion of gelatin with a neutral salt of Li, K, or NH_4, when followed by a perfusion with distilled water, causes an additional excessive swelling; while the perfusion of the powdered gelatin with neutral salts of the bivalent cations, Mg, Ca, Sr, and Ba, does not cause any additional swelling when the

gelatin is afterward perfused several times with distilled water. It would, therefore, follow that gelatin treated by neutral salts with bivalent cations increases its mass of gelatin ions either not at all or only to a negligible extent. This is apparently the case. When 2 gm. of powdered gelatin are first perfused twice with M/8 $CaCl_2$ and are then perfused three times with 25 cc. of distilled water, and if then a 3 per cent gelatin solution is prepared, 5 cc. of such a solution are precipitated by slightly less than 5 cc. of alcohol (at room temperature), which is approximately the same figure as that for gelatin not treated with salt. The gelatin is, therefore, apparently not ionized by Ca.

5. This difference in the action of neutral salts with bivalent and univalent cations, namely, that the latter ionize the gelatin while the bivalent cations will apparently not form ionizable compounds, is the basis of the antagonistic action between the two types of salt. When we treat powdered gelatin with a mixture of NaCl and $CaCl_2$ the addition of a comparatively small amount of $CaCl_2$ to M/8 NaCl will inhibit the swelling which would follow if the gelatin were perfused first by the NaCl alone and then by distilled water. It can be shown that when we perfuse 2 gm. of powdered gelatin twice with 25 cc. of 50 cc. M/8 NaCl + 4 cc. M/8 $CaCl_2$, and then three times with distilled water, the gelatin is precipitable by alcohol; while if we use the pure M/8 NaCl or 50 cc. M/8 NaCl + 1 or 2 cc. of M/8 $CaCl_2$ the 3 per cent gelatin solution freed from the salt is not precipitable by alcohol. We, therefore, come to the conclusion that the phenomenon of swelling of gelatin under the influence of neutral salts with univalent cation and the inhibition of this swelling by neutral salts with bivalent cation (alkaline earths) are due to the fact that the former salts cause the formation of ionizable gelatin salts, while the latter apparently cause the formation of non-ionizable gelatin salts.

II.

We may raise the question: Which of the possible cases of ionization actually occurs when gelatin is treated with neutral salts with univalent cation, like NaCl? If we assume that the gelatin molecule combines with both anion and cation the neutral complex gelatin$\begin{matrix}\diagup\text{Na}\\ \diagdown\text{Cl}\end{matrix}$ can give rise to three types of protein ions:

(1) $\text{Gelatin-Cl} + \text{Na}^+$

(2) $\text{Gelatin-Na} + \text{Cl}^-$

(3) $\text{Gelatin} + \text{Na}^+ + \text{Cl}^-$

In (1) gelatin ion would be negative, in (2) gelatin ion would be positive, and in (3) the gelatin ion would have both a negative and a positive charge in different parts of the molecule. We shall see that the facts speak in favor of (1); namely, an exclusive or a prevailing formation of negative gelatin ions. According to our assumption the additional swelling of gelatin due to ionization should begin when the salt solution used for perfusion to wash out the M/8 NaCl is so dilute as not to suppress the electrolytic dissociation of the gelatin-salt compound completely. Since this suppression depends upon the common ion of the gelatin-salt compound and the salt, it should be possible to ascertain which this common ion is, the anion or the cation of the salt. If, for instance, the dissociation of the gelatin salt occurs according to (1) into gelatin + Na^+, the lowest concentration of Na_2SO_4 which just allows the additional swelling of a gelatin previously treated with M/8 NaCl should be about one-half of that in which the swelling begins when lower concentrations of NaCl are used; since, *e.g.*, a M/256 Na_2SO_4 solution sends about twice as many Na ions into solution as does a M/256 NaCl solution. In other words, that concentration of a salt which is just able to inhibit completely the subsequent swelling of gelatin previously treated with M/8 NaCl should depend only upon the concentration of the cation of this salt, if the type (1) of the dissociation of the gelatin salt exists; namely,

(1) $\text{Na-gelatin} \rightarrow \text{gelatin} + \text{Na}$

This is the case. We know from Table I that the inhibiting concentration of NaCl for the additional swelling[11] of gelatin previously treated with M/8 NaCl is M/64 NaCl. In this concen-

[11] By additional swelling we mean the swelling which takes place as a consequence of the previous treatment with M/8 NaCl, when the excess of the salt is removed and the ionization of the gelatin salt no longer too much depressed.

tration an increase of 5 mm. in the height of the cylindrical mass of gelatin will show itself. With a further dilution of NaCl the swelling of the gelatin increases very rapidly.

We now undertook the following series of experiments. We perfused 2 gm. of powdered non-bleached gelatin, as in the previous experiments, first with 50 cc. M/8 NaCl, and followed this with a perfusion with solutions of different neutral salts of increasing dilution. Table II gives the results of such a series of experiments.

TABLE II.

	Additional swelling of 2 gm. powdered gelatin previously perfused twice with 25 cc. M/8 NaCl and three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaCl	0	0	2.5	**6**	15	21	33.5	42	48			54
NaBr	0	2.0	2.0	**5**	12.5	17.5	27	43	42	46	53	52.5
$NaNO_3$	1	?	4	**7**	12.5	19.5	29.5	39	43.5	51	49	54
Na acetate	1.5	1.5	3	**6.5**	15	22	31	38	45.5	44	48	55
NaCNS		3.5	4	**7**	12	21	31	40	46	53	58	60
Na_2SO_4	1	1.5	2.5	3.5	**8**	14	22.5	30	39	47	55	54
Na_2 oxalate	2	2	2	3.5	**6.5**	14.5	24	29	35	46	50	56
Na_2 tartrate	2	2	2	3	**5.5**	13.5	23	32.5	43	46	52.5	56.5
Na_2 malate	0	1	1	2.5	**5**	13	20	31	35	39	43	51

While the sodium salts with monovalent anion all show the beginning of additional swelling (*i.e.* swelling amounting at least to 5 mm. increase in height of column) in a dilution of M/64 the sodium salts with bivalent anion show this increase in twice this dilution; namely, M/128. This would indicate that the metal ion alone or prevailingly determines the degree of electrolytic dissociation of the gelatin sodium salt. In other words, the gelatin salt formed, when gelatin is treated with NaCl, dissociates exclusively or prevailingly in the form gelatin $+ Na.^{+}$ The anion plays obviously a minor if not a negligible part.

When we test 3 per cent solutions of such gelatin with alcohol we find that the solution always becomes non-precipitable as soon as the swelling becomes marked.

When we treat gelatin first with M/8 NaCl and then with dilute solutions of salts of other monovalent cations the results should be essentially the same as if we treated the gelatin with solutions of NaCl, since a compound gelatin-Na should exchange its metal with Li or K or NH_4 without essential molecular alteration. Hence the results should remain practically the same if a treatment with M/8 NaCl is followed by solutions of NaCl or of LiCl or KCl. This is true, as Table III shows.

TABLE III.

	Additional swelling of powdered gelatin perfused twice with 25 cc. M/8 NaCl and three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
LiCl	1	1.5	3.5	**6**	10.5	15	24.5	34.5	44.5	52	53.5	57.5
KCl	1	2	2.5	**6.5**	11.5	16	24	33	38.5	44	46.5	53.5
NH_4Cl	2	2	2	**5**	13	23	22.5	39.5	48	47		56
Li_2SO_4	3	0	2	2.5	3.0	**10**	19.5	30.5	45.5	51	51	60
K_2SO_4	1	1.5	0.5	3.0	**6.5**	11	20	29	40.5	42	50.5	54.5
$(NH_4)_2SO_4$	1.5	1.5	2	3	**6**	12	18	26	42	50	45	53
K_2 oxalate	1	1	1.5	3.5	**7.5**	17	22.5	25	33.5	43	46.5	55
$(NH_4)_2$ oxalate	0	1	1	2.5	**5**	11.5	22.5	27	36.5	48.5	49	54
K_2 tartrate	0	0	1.5	1.5	**7**	13	19	28	37	42	46	53
$(NH_4)_2$ tartrate	2	1.5	2	5 ?	**7**	16.5	22	35.5	43.5	49.5	52	55

For the chlorides the swelling becomes noticeable at a dilution of M/64, for the sulfates, oxalates, and tartrates at nearly twice the dilution; namely, about M/128. Only Li_2SO_4 is an apparent exception for reasons still to be investigated.

It was shown by special controls that if we first treat powdered gelatin with M/8 Na_2SO_4 and follow this by weaker concentrations of Na_2SO_4 we get the same critical value for the commencement of swelling as when we first treat the gelatin with M/8 NaCl and follow this with weaker concentrations of Na_2SO_4; namely, M/128. M/8 LiCl followed by different concentrations of LiCl gives the same critical value for the commencement of swelling as M/8 NaCl followed by different concentrations of LiCl.

If the anion has a distinct effect it must show itself in com-

paring the effects of washing with $MgCl_2$ and $MgSO_4$ and of $CaCl_2$ and $CaSO_4$. When we perfuse powdered gelatin first with M/8 NaCl and then with $CaCl_2$ solutions of a higher degree of dilution we must expect a lower limit of concentration where the swelling begins, since $CaCl_2$ by replacing the NaCl in combination with gelatin transforms the ionizable gelatin into a less ionizable or otherwise modified complex. The concentration of $CaCl_2$ must therefore become comparatively low before swelling becomes possible. If the additional swelling, *i.e.* the ionization of the gelatin, depends on the cation alone or predominantly, $CaSO_4$ and $MgSO_4$ should have the same critical concentration for the commencement of the swelling, which is the case, as Table IV shows. The critical concentration for all the salts is M/512.

TABLE IV.

	Additional swelling of powdered gelatin perfused twice with 25 cc. M/8 NaCl and then three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
$MgCl_2$	0	0	0	0	0	4	13	26	42	49	53	52
$MgSO_4$	1.5	0	0	−1	0	1	7.5	22.5	38	48	55	63
$CaCl_2$	0	0	0	0	0	1.5	8	24	39	48	51	47
$CaSO_4$					−3.5	−2.5	+8.5	18.5	37.5	43	47	55
$SrCl_2$	0	0	0	0	0	1	8	18	36	43	52	52
$BaCl_2$	0	0	0	0	0	1	10	21	40	45	51	50

All these facts support the idea that neutral salts form compounds with gelatin which dissociate into a positive metal ion (that of the salt used) and into a negatively charged protein ion which may or may not contain the anion of the salt. This idea was tested by experiments on the migration, in an electric field, of gelatin treated with M/8 NaCl and then freed from the salt by washing. Such gelatin migrates to the anode as our theory demands.

III.

The idea that only the cation enters into the ionization of gelatin might be interpreted to mean that a reaction of the following kind occurs between gelatin and NaCl:

$$RCOOH + NaCl \rightarrow RCOO\text{–}Na + HCl.$$

In this case the supernatant solution should have an acid reaction. This was not the case.

Pauli makes another suggestion based on the fact that proteins in general are stronger acids than bases, and that hence they must undergo a stronger hydrolytic dissociation where they act as base than where they act as acid. If such a protein combines with NaCl the Cl would undergo hydrolytic dissociation and be washed away as free HCl. But if this were the case, the water with which NaCl-gelatin is washed should have an acid reaction. Although we were not able to demonstrate such an acid formation, we should remember that little acid was formed at the utmost and that some of it may have been bound again by gelatin molecules. The important fact is that under the influence of a neutral salt, of the type NaCl, the gelatin forms a sodium gelatinate, which dissociates electrolytically into a negatively charged gelatin ion and a positively charged metal ion—that of the salt used.

This assumption is further supported by hydrogen ion determinations which Dr. Dernby has made in my laboratory and which I incorporate here with his permission. The writer had shown in a previous paper that pig's bladder behaves like powdered gelatin inasmuch as it shows a considerable additional swelling in H_2O when previously treated with NaCl or any neutral salt with univalent cation; while no such additional swelling in H_2O occurs after previous treatment with neutral salts with bivalent cation. Dr. Dernby found that when the membranes treated with M/8 NaCl (or KCl, etc.) were afterward put into distilled water the latter became slightly alkaline; but that this was not the case when the pig's bladder was previously treated with $CaCl_2$.

Dr. Dernby determined the hydrogen ion concentration by Sörensen's colorimetric method, using his standard phosphate solutions and neutral red as indicators. In all these experiments the hydrogen ion concentration changes in the same way as the swelling. Where a strong additional swelling occurs, as in H_2O after a previous treatment in NaCl, the hydrogen ion concentration diminishes; where no additional swelling occurs no change

in the hydrogen ion concentration is observed. In the M/8 NaCl solution where the membrane does not swell no change in the hydrogen ion concentration occurs.

Dr. Dernby's method was as follows. Pieces of pig's bladder of equal weight (about 0.75 gm.) were put into 50 cc. of a salt solution for 1 hour, then washed repeatedly, and transferred into 50 cc. of distilled water (except in the controls, where the bladder remained permanently in distilled water or in the salt solution).

Table V gives his results.

TABLE V.

After	Hydrogen ion concentration of a solution containing pig's bladder treated as stated (expressed with Sörensen's symbol pH).					
	Control in distilled water.	In M/8 not washed.	In M/8 NaCl washed in H_2O after 1 hr.	KCl	NH_4Cl	Na_2SO_4
hrs.						
0	About 6.4	6.4	6.4	6.2 to 6.4		
½	6.4	6.4	6.4			
1	6.4	6.4	6.4	Washed.		
2	7.0	6.4	7.1	6.8	6.8	6.9
3	7.1	6.6	7.2	7.0	7.0	7.0
5	7.1	6.6	7.3	7.3	7.3	7.3
24	7.1	6.9	7.4	7.3	7.3	7.3

In these experiments the distilled water and salt solutions contained CO_2 and this is the reason why at the first reading the H ion concentration is so high (6.2 to 6.4 instead of 7). The determinations prove that the solutions become more alkaline when the pig's bladder was first treated with a neutral salt with monovalent cation. This agrees with our assumption of an electrolytic dissociation into a negative gelatin ion and a positive Na^+ ion, since slight hydrolytic dissociation would lead to the formation of a stronger base (NaOH) and a weaker acid, gelatin-COOH.

We had shown that the bivalent metals, Mg, Ca, Sr, and Ba, form in all probability less ionizable compounds with gelatin. Hence we should expect that a previous treatment of pig's blad-

der with Ca should not increase the alkalinity of the distilled water. This is actually found to be the case by Dr. Dernby (Table VI).

TABLE VI.

After	Hydrogen ion concentration of a solution containing pig's bladder treated as stated (expressed with Sörensen's symbol pH).			
	Control in distilled water.	In M/8 $CaCl_2$ 1 hr. then in distilled water.	M/8 $MgCl_2$	M/8 $SrCl_2$
hrs.				
0		6.4		
½		6.4		
1	About 6.5	6.4		
		Then transferred into distilled water; immediately after transferring the H ion concentration was in all cases the same pH = 6.2 to 6.4.		
3	6.7	6.4	6.5	6.5
5	7.0	6.6	6.7	6.7
24	7.1	6.9	6.9	6.9

It is obvious that pig's bladder previously treated with solutions of $MgCl_2$, $CaCl_2$, and $SrCl_2$ does not cause a change of the same order in the reaction of the water as was found for bladder treated with NaCl and NH_4Cl. This harmonizes with the assumption that the bivalent metals form non-dissociable or less dissociable gelatin salts.

IV.

In the previous note[8] the writer has stated that the additional swelling caused by a treatment with NaCl was observed in powdered gelatin and in pig's bladder, but not in solid blocks of gelatin. It was found that the cause of this difference is the fact that it is easy to ionize large masses of powdered gelatin with a salt and then wash the unnecessary salt solution away, while this is difficult if not impossible with solid blocks of gelatin. The salt solution apparently diffuses slowly in and out of the block. For this reason the following modification of the method was adopted. Powdered gelatin was treated with a M/8 NaCl solu-

tion in the usual way, and the salt solution was then removed by washing the powdered mass three or four times with distilled water. After all the water had run off the mass of gelatin was poured into a beaker and completely liquefied by heating for about 10 minutes in a water bath of about 40°. This homogeneous liquid mass of gelatin was then put into a bag of collodion and exposed to the draft of an electric fan for about 24 hours, when it had lost most of the water. When such blocks of gelatin, previously treated with M/8 NaCl and freed from the NaCl solution by repeated washings with H_2O, were put into H_2O they showed the same excessive swelling as had been observed in the case of the powdered gelatin; while this excessive swelling was prevented when the block of gelatin previously treated with M/8 NaCl was put back into M/8 NaCl.

The following is an example. Into each of three cylindrical funnels were put 2 gm. of finely powdered Cooper's unbleached gelatin. The gelatin in one funnel (I) was perfused six times in succession with distilled water. The second (II) was perfused twice with 25 cc. M/8 NaCl, then four times with 25 cc. of distilled water; the third (III) was perfused six times with 25 cc. M/8 NaCl. After all the water had run out, the weight of the first mass of gelatin was 34 gm., that of the second, 59.15 gm., and of the third 24.4 gm. The previous treatment with salt increased the swelling from 32 to 57 gm. Incidentally the reader will see that contrary to Hofmeister's statement the gelatin did not swell more when permanently treated with M/8 NaCl than it did when permanently treated with H_2O, since in the latter case it absorbed 32, in the former only 22 gm.—of which part was NaCl.[12] These three lots of gelatin were then put into collodion bags and exposed to the fan until their respective masses were 8.4 (I), 7.4 (II), and 6.05 (III) gm. The three masses of gelatin were then liquefied by heating to about 40°C., poured into a flat Petri dish, allowed to gelatinize, and then blocks of equal size were stamped out of each; the respective weights of the three blocks were 7.6 (I), 6.04 (II), and 4.19 (III) gm. I and II were put into distilled water, III was put into M/8 NaCl. After 24 hours the weighing gave the following result:

[12] Ash determination showed that the gelatin contained in this case the same proportion of NaCl and water as did the M/8 NaCl solution.

I	II	III
22.02 gm.	52.8 gm.	12.9 gm.

The gelatin treated first with M/8 NaCl swelled after removal of the salt two and one-half times as much in distilled water as did the gelatin not treated previously with NaCl. We are, therefore, justified in saying that blocks of gelatin behave in the same way as does powdered gelatin if only care is taken that the excess of salt is removed from the block before it is exposed to the distilled water. If a block of gelatin is first put into M/8 NaCl and then into distilled water, we get different results on account of the slowness of the diffusion of the salt into and out of the block.

In experiments with powdered gelatin we had found that the valence of the cation was much more effective than the valence of the anion. A previous treatment of powdered gelatin with M/8 $CaCl_2$ (or $MgCl_2$ or $SrCl_2$ or $BaCl_2$) did not cause the excessive swelling which was caused by M/8 NaCl, and it was found that the addition of a small amount of $CaCl_2$ to NaCl stopped the after-effect of NaCl upon the subsequent swelling of gelatin in H_2O (antagonistic salt action). The same is true for blocks of gelatin as the following experiment shows.

2 gm. of powdered gelatin were put into each of three cylindrical funnels. Funnel I was perfused six times with 25 cc. H_2O; funnel II twice with 25 cc. M/8 $CaCl_2$ and then four times with 25 cc. H_2O; and funnel III six times with 25 cc. M/8 $CaCl_2$. The next day (after all the water had drained off) the weight of the gelatin liquefied by heating to about 40° was

I	II	III
27.8 gm.	25.8 gm.	24.3 gm.

The $CaCl_2$ treatment had, therefore, little effect upon the swelling of powdered gelatin. The mass of the three lots was then reduced by evaporating in a collodion sac to 7.6, 7.1, and 7.3 gm. each and blocks of (I) 6.2, (II) 6.0, and (III) 4.7 gm. were cut out. Blocks I and II were put into distilled water, and block III into M/8 $CaCl_2$. After 41 hours the weight was as follows:

I	II	III
25.4 gm.	24.1 gm.	17.3 gm.

Comparing I and II we notice that the previous treatment with $CaCl_2$ did not increase the swelling of the block of gelatin.

In the same way all the experiments on antagonistic salt action described in the previous paper[8] could be repeated with solid blocks of gelatin.

These and other experiments allow us to state that the influence of salts upon the swelling of powdered gelatin published in the previous paper holds for solid blocks of gelatin also if only care is taken to remove the excess of salt from the block. The results obtained on powdered gelatin can, therefore, be utilized for the theory of swelling of gelatin in general.

V.

Our results contradict the conclusions which are drawn by many authors from the old experiments of Hofmeister[13] on the influence of neutral salts and sugar on the swelling of gelatin. Hofmeister stated that the salts, according to their effect upon the swelling of gelatin, may be divided into two groups. The one group makes the gelatin swell more than distilled water, the other makes it swell less than distilled water. The former group includes NaBr, $NaNO_3$, NH_4Cl, NaCl, KCl. The second group includes the acetates, citrates, tartrates, and sulfates; but also alcohol, grape sugar, and cane sugar. From these observations it has generally been concluded that the anion is the decisive factor for the swelling; and that Hofmeister's results agree with the influence of salts on other qualities of proteins. Such a conclusion overlooks Hofmeister's distinct statement that alcohol and sugars act like acetates and tartrates. This fact is of importance since it confirmed Hofmeister in his idea that the attraction of the salt for water was one of the factors by which salts and sugars influence the swelling of gelatin. Thus he states that the sulfates, tartrates, citrates, and acetates, attract water more powerfully than chlorides or bromides and hence prevent the gelatin from absorbing as much water as it does in pure water or in the presence of chlorides.

Hofmeister's method cannot give any clear idea concerning the influence of salts on the swelling of gelatin since this influence depends chiefly upon the ionization of the gelatin. Two proc-

[13] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 210.

esses are necessary to obtain the correct estimate of the effect of this ionization; first, treatment of the whole mass of gelatin with a sufficiently high concentration of the neutral salt (M/8 or M/4), and such a treatment can only be effective if the protein is in finely divided condition; and second, removal of the excess of salt in order to permit the electrolytic dissociation of the protein. Neither condition is fulfilled in Hofmeister's method. This also explains why he got similar results with sugar and alcohol as with sodium acetate and sodium sulfate. The differences Hofmeister observed in the action of different salts are comparatively slight and they cannot be used for a theory of the action of salts on swelling.

Lenk[14] tried to demonstrate the antagonistic action between NaCl and $CaCl_2$ on gelatin. He used Hofmeister's method, trying to show that blocks of gelatin swell less in NaCl solution when $CaCl_2$ is added, but the effects observed are small and, as the writer believes, within the limits of error of such experiments. Fenn[15] tried to show that gelatin solutions require less alcohol for precipitation in the presence of NaCl when $CaCl_2$ is added. This is a much more promising method for the study of antagonistic salt action than the one used by Lenk, but the results of Fenn published in his preliminary notices show that he also studied the effects of salts on protein without first removing the excess of salt so that he missed the ionization effect.

Theoretical Remarks.

The paper gives a new and convenient method for the investigation of the effects of electrolytes on the physical properties of proteins and other so called colloids. This method leads to entirely different results from those obtained by the old method of Hofmeister on the swelling of gelatin, and this difference is due to the fact that in Hofmeister's method the effect of the salt on swelling is observed in the presence of an excess of salt, which, as our method shows, inhibits the additional swelling effect of the salt. This latter is the only characteristic effect of the salt on the swelling. Hence it is not possible to discover by Hofmeister's

[14] Lenk, E., *Biochem. Z.*, 1916, lxxiii, 15, 58.
[15] Fenn, W. O., *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539.

method the true characters of the effect of neutral salts (or any true effect of salts) upon the swelling of gelatin.

The most important result obtained with our method is the proof that the influence of salts upon the swelling of gelatin is of a stoichiometrical character; *i.e.*, we can utilize the limiting concentration of different neutral salts for the additional swelling of sodium gelatinate to ascertain the molecular concentration of the salt. Experiments on the action of salt upon gelatin treated previously with acid or alkali harmonize with the results given in this paper. They will be discussed in a following paper. The fact that dried pig's bladder behaves similarly to the powdered gelatin indicates the more general character of our results.

In the explanation of these phenomena the writer has adopted the idea that it is the degree of ionization of gelatin salts (of the type sodium gelatinate) which determines the additional swelling observed when the gelatin is first treated with a high concentration of an alkali metal salt (M/8 or M/4) and the salt has been washed away. The reasons for this assumption are given in the paper and need not be repeated here; but we may add that the new method and the new stoichiometric facts do not depend upon this hypothesis. If we adopt the ionization hypothesis, which seems supported by the facts known at present and contradicted by none, it follows, from our observations that we have at present two cases in which antagonistic salt action is clearly due to the fact that one electrolyte, *e.g.* NaCl or acid, causes the formation of ionized protein, while the other electrolyte, *e.g.* $CaCl_2$ or neutral salts in general, causes the transformation of ionized into non-ionized protein, or inhibits in some other way the swelling effect of ionization of the protein molecule. The observations on the influence of neutral salts on the diffusion of potassium salts into the egg of *Fundulus* may form a third case.

SUMMARY.

1. A new method has been described which allows us to study the effect of neutral salts on gelatin. The essential part of this method consists in using the protein in powdered form, applying the salt in not too low a concentration (M/8 or M/4), and then washing away the salt solution.

2. This method has led to the result that the influence of neutral salts on the swelling of gelatin is of a stoichiometric order. Powdered gelatin, when perfused by M/8 or M/4 solution of NaCl, shows an additional swelling when afterward perfused by a weaker solution of a neutral salt with univalent metal. This *additional* swelling is only possible as long as the weaker solution remains below a certain concentration. A comparison of this critical concentration for neutral salts of univalent cations with univalent and with bivalent anions shows that the limiting molecular concentration for the *additional* swelling is twice as great if the anion is univalent as when it is bivalent; regardless of the nature of the anion and cation. The facts can be best explained on the assumption that the inhibiting effect of the salt upon the additional swelling is due to the diminution of the degree of electrolytic dissociation of a metal-protein compound. If we make this assumption, which is supported by the facts known at present, our observations lead to the following conclusions.

3. Neutral salts with a univalent cation (in concentrations of M/8 or M/4) form highly ionizable salts with gelatin.

4. It seems that these gelatin salts ionize under formation of a positively charged metal ion (that of the salt used) and a negative gelatin ion which may or may not contain the anion of the salt in non-dissociated bondage.

5. The formation of these gelatin ions causes the considerable additional swelling when the gelatin is first treated with the salt and then, after the salt is washed away, is exposed to distilled water.

6. The metals of the alkaline earth group form salts with proteins of the type calcium gelatinate, which are not capable of swelling and perhaps little or not at all of ionization. The transformation of protein salts with univalent cation (type sodium gelatinate) capable of swelling into protein salts with bivalent cation (type calcium gelatinate) not capable of swelling is the cause of the antagonistic action of the metals of the calcium group.

7. These results contradict the conclusions drawn from Hofmeister's experiments on the swelling of gelatin and it is pointed out that he was misled by a method not suited for the purpose.

A STUDY OF THE NON-PROTEIN NITROGEN OF WHEAT FLOUR.*

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Preliminary to a projected investigation concerning the more important biochemical changes which occur when wheat is frosted, as well as the relation of these changes to bread-making value, and particularly in order to study the effect of premature freezing on the nitrogen compounds of the wheat kernel, it has been found desirable to develop a more satisfactory method for the separation of protein from non-protein nitrogen compounds in wheat flour than an examination of the literature has revealed. A number of methods have been developed for the separation of protein from non-protein nitrogen in almost all kinds of biological products, and several of these appear to be satisfactory for their purposes. With cereals, however, none of these methods seems to answer the purpose since cereals contain alcohol-soluble proteins, which are not encountered in any other plant or animal tissues.

Reagents ordinarily used for precipitating proteins, such as alcohol, acetic acid, trichloroacetic acid, salts of heavy metals, colloidal iron, aluminum hydroxide cream, phosphotungstic acid, and tannic acid, are for various reasons unsatisfactory for removing gliadin from water extracts of flour.

Ritthausen,[1] 40 years ago, advocated the quantitative removal of the proteins from milk, by alternately adding to the protein in solution dilute copper sulfate and potassium hydroxide until the proportions were such that the copper precipitate would no longer redissolve. The insoluble copper-protein compounds were then

* Published with the approval of the Director of the Montana Agricultural Experiment Station.

[1] Ritthausen, H., *J. prakt. Chem.*, 1872, v, 215.

removed by filtration. This principle in modified forms has since been made use of by different investigators, although the accuracy of the separation of protein from non-protein nitrogen by this method has always been questioned.

Osborne and Leavenworth[2] have recently reported a study of copper-protein compounds, using edestin and gliadin in their experiments, and have found that if the correct amount of copper sulfate is added to a solution of gliadin in dilute sodium hydroxide solution, the gliadin and copper are both practically completely precipitated. As they state, however, there are several points to be definitely cleared up if this procedure is to be made a basis for the accurate separation of protein from non-protein nitrogen compounds in extracts from biological material.

When the principle of Osborne's procedure was tried with flour extracts in this laboratory, it was found that more nitrogen was removed by this means than by the use of any other of the reagents previously mentioned. Phosphotungstic acid removed nearly, but not quite, as much and tannic acid slightly less. Moreover, the copper-protein precipitate filtered readily, giving a water-clear solution, which could easily be concentrated under reduced pressure to one-twentieth of its original volume. That there was probably no copper in the filtrate, other than that which was in combination with amino-acids and peptides, was indicated by the fact that no blue color was perceptible, nor were positive tests with potassium ferrocyanide obtained until after considerable concentration. The method seemed so simple of manipulation that it was decided to test the effectiveness of the separation by ascertaining whether or not all protein was removed, as well as to test the copper-protein precipitate for free amino nitrogen as an indication of the presence or absence of less complex nitrogen compounds in the copper-protein precipitate.

In Table I are data which indicate the total nitrogen which is *not* precipitated from a water extract of a standard patent flour (Ceretana) by the copper method, as compared with the total nitrogen *not* removed from the same extract by tannic acid, colloidal iron, and phosphotungstic acid, respectively. The figures represent averages of several determinations by each method.

[2] Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1916-17, xxviii, 109.

TABLE I.

Method of treatment using 50 cc. portions of extract.	Total N not precipitated.
	gm.
1. 1 cc. Merck's 5 per cent colloidal iron ppt. at room temperature, followed by 1 cc. of concentrated $MgSO_4$ solution	0.00266
2. Same as No. 1 with precipitation at boiling temperature	0.0033
3. Precipitation with 10 cc. of 10 per cent phosphotungstic acid after making strongly acid with HCl	0.0010
4. Precipitation with tannic acid	0.0012
5. Copper method	0.0008

The details of the copper method as used on flour extracts in this laboratory are presented in the following experiments.

Experiment I.

Ceretana, a standard patent flour milled by the Bozeman Milling Company of Bozeman, Montana, was used in the preliminary work, which consisted of determining the proper proportions of flour to water for the extraction, the length of time of extraction, and a comparison of the copper method with the other methods mentioned earlier in this paper with respect to the "total non-protein nitrogen" determined by other methods. Distilled CO_2-free water, saturated with toluene, was used for all extractions. After trying various proportions of flour to water, 20 parts of water to 1 of flour was decided upon as a convenient proportion to use for extractions. Extractions were carried on for varying lengths of time, using portions of 20 gm. of flour to 400 cc. of water in 500 cc. Erlenmeyer flasks. The flasks were shaken vigorously every 15 minutes, for periods of 2, 3, 4, 5, 6, and 12 hours, respectively. At the end of each period the extract was filtered through paper, the proteins were precipitated by treating 50 cc. of extract with 15 cc. of 0.1 N NaOH, followed by 16 cc. of 0.1 N $CuSO_4$, and total nitrogen was determined in the filtrate from the copper precipitation. The amount was found to be the same for the 2 hour extraction as for the 12 hour period, and therefore a minimum extraction period of 2 hours was adopted. Perhaps the most satisfactory method for the

determination of "*total* non-protein nitrogen" in flour extracts by the above method is to pipette 100 cc. of flour extract into a 200 cc. sugar flask, add 25 cc. of 0.1 N NaOH followed by 27 cc. of 0.1 N $CuSO_4$, shake vigorously several times until a water-clear supernatant liquid remains after the precipitate settles, make to the mark, filter, and determine nitrogen by the Kjeldahl method in 100 or 150 cc. of the filtrate.

The effectiveness of the method was then examined by studying the nature of the nitrogen compounds left in the filtrate from the copper-protein precipitation. In this work, two flours, A and B, were used. Flour A was the Ceretana patent flour mentioned previously, while B was a flour milled from some slightly frosted Karkov wheat grown in Montana. 1 liter portions of filtered flour extract were placed in 2 liter flasks, and 400 cc. portions of 0.1 N NaOH were added, followed by 400 cc. of 0.1 N $CuSO_4$. Then small portions (of about 10 cc. each) of 0.1 N $CuSO_4$ were added, the whole being well shaken and allowed to settle after each addition, until an absolutely clear, colorless supernatant liquid remained after the precipitate settled. *It appears to be absolutely necessary that the 0.1 N alkali be kept in paraffin-lined containers, or else be made up fresh for each occasion,* as it was found that alkali containing dissolved glass would neither cause a sharp separation nor give a clear, colorless filtrate. Finally, distilled water was added up to the 2 liter mark, and the filtration performed on a large paper filter. The filtration proceeds rapidly and easily when the precipitation is made in the manner described. The filtrate was then slightly acidified with acetic acid and concentrated under diminished pressure to one-twentieth of its volume. The concentrated solutions were slightly viscous on account of dissolved carbohydrate material, and gave a slight test for copper with ferrocyanide, which they did not give before concentration. They also showed the slightest trace of color due to copper salts. These concentrated solutions are referred to in the remainder of this paper as Solution X. These solutions gave no perceptible biuret reaction of any kind, but did give a very faint Millon's reaction, and also a slight Adamkiewicz' reaction. The latter two reactions are indicative of the presence of the amino-acids, tyrosine and tryptophane, but do not necessarily show that they are in protein combination. Solution X

gave a slight color with Nessler's reagent, indicating but a trace of free ammonia. Amino nitrogen was determined in Van Slyke's micro-apparatus using 5 cc. portions of Solution X. Amide nitrogen was determined by diluting 50 cc. of Solution X to 100 cc., boiling for 2 hours with 2.5 cc. of concentrated sulfuric acid, according to the method of König,[3] and distilling with an excess of calcium hydroxide under reduced pressure. In order to find out approximately the nature of the rest of the nitrogen in the extracts, 25 cc. portions of Solution X were hydrolyzed with strong hydrochloric acid for about 12 hours, after which ammonia and amino nitrogen were again determined, and the increase in these constituents over the amounts as determined before hydrolysis was considered as indicative of the nature and complexity of any nitrogen compounds still present in peptide or protein form. In Table II are presented results of analyses of flours A and B.

TABLE II.

Constituent determined.	Flour A (sound patent).	Flour B (slightly frosted).	Flour A.	Flour B.
	per cent	*per cent*	*gm.*	*gm.*
Total N of flour	1.89	1.79		
Per cent of total N of flour extracted by distilled CO_2-free water in $2\frac{1}{2}$ hours	16.06	15.40		
Per cent of total flour N not precipitated by copper	1.62	2.12		
Free ammonia in Solution X	Trace.	Trace.		
Amide N in total "non-protein" N	20.00	26.12		
Gm. of amide N in 100 gm. of flour			0.006	0.0095
α-Amino N in total "non-protein" N	6.62	7.79		
Gm. of amino N in 100 gm. of flour			0.002	0.003
Ammonia N after hydrolysis	10.91	10.32		
α-Amino N increase due to hydrolysis	19.34	20.22		
Humin N after hydrolysis	8.16	7.37		
Residual nitrogen by difference	34.97	28.18		

It will be observed from Table II that the actual quantity of free amino nitrogen in a normal flour is exceedingly small, being about 2 mg. for every 100 gm. of flour. There is about three

[3] König, J., Chemie der menschlichen Nahrungs- und Genussmittel, Berlin, 4th edition, 1910, iii, 274.

times as much nitrogen in the acid amide form. The figures for amino nitrogen vary widely from those obtained by Swanson and Tague[4] who determined amino nitrogen in a patent flour using Sörensen's formol titration method and report about nine times as much as is found in these experiments. They used flour extracts without attempting to remove the proteins. Van Slyke micro determinations were made directly on some flour A extract in this laboratory in order to obtain a more satisfactory comparison with their method. The presence of the proteins made it impossible to take an adequate concentrated sample so that determinations had to be made using 5 cc. of the extract, corresponding to 0.25 gm. of flour. This, according to the method used by Swanson and Tague, should give about 0.1 cc. of nitrogen gas which may be easily measured in the micro-apparatus. In no case, however, could more than 0.02 cc. be obtained, while the average of four determinations was about 0.01 cc., which is too small an amount to be considered for the purposes of a satisfactory determination, although quite in accordance with the findings using the Van Slyke method on the concentrated extract from which the proteins had previously been precipitated with copper; *i.e.*, Solution X.

Table II shows an increase in ammonia and amino nitrogen in Solution X after prolonged hydrolysis with strong acid. The increase, however, is not great enough to indicate the presence of any considerable amount of protein. It suggests, however, that there is some nitrogen in the form of peptides which escapes precipitation by the copper method, although it does not preclude the possibility that traces of protein may still be present also. Hart and Bentley,[5] studying the non-protein nitrogen of some feeding-stuffs, found that after treatment with Stutzer's reagent some nitrogen of the nature of peptide linkings remained in solution. The same seems to be true when Osborne's procedure is followed, although it is probable that the precipitation of the true proteins is practically complete if the proper manipulation is followed.

[4] Swanson, C. O., and Tague, E. L., *J. Am. Chem. Soc.*, 1916, xxxviii, 1098.

[5] Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii. 477.

Experiment II.

In order to obtain a more satisfactory idea of the nature of the peptide linkings left in solution after the copper treatment, a portion of Solution X from flour A was acidified and treated with phosphotungstic acid. A considerable precipitate formed which contained 40.91 per cent of the total nitrogen in the solution notwithstanding the fact that when the *original water extract* of flours is treated with phosphotungstic acid no more (and often less) nitrogen is precipitated than by the copper method. However, when Solution X was subjected to a further treatment with copper, as in the first removal of the proteins, no more nitrogen was precipitated. Furthermore, when a portion of Solution X was mixed with about nine times its volume of 95 per cent alcohol, 30 per cent of the total nitrogen in the solution was precipitated, along with the dextrins and other carbohydrates insoluble in alcohol. These facts strongly suggest the presence of peptide linkings of a less complex nature than true protein. The phosphotungstic acid precipitate was filtered off, washed with a solution of phosphotungstic and hydrochloric acids, and hydrolyzed for about 12 hours with strong hydrochloric acid, after which ammonia and amino nitrogen were determined. Amino nitrogen was also determined in the filtrate from the phosphotungstic acid precipitation. Although the determinations were made on small amounts of material, they were confirmed by several closely agreeing duplicates and blanks on each analysis, the result being reported in Table III.

TABLE III.

Constituent determined in terms of total N in Solution X.	Flour A.
	per cent
N precipitated by alcohol	30.00
N precipitated by further treatment with $CuSO_4$ and NaOH	None.
N precipitated with phosphotungstic acid	40.91
Total amino N in Solution X	6.62
Total amino N in filtrate from phosphotungstic acid precipitation	4.81
Amino N from exposed amino groups of peptides (by difference)	1.81
Ammonia N after hydrolysis of phosphotungstic acid precipitate	5.71
Amino N after hydrolysis	14.30

As nearly as may be ascertained from determinations with such small amounts of material as are necessitated in the preceding experiments, the figures reported in Table III show that there cannot have been much, if any, protein left in solution after the first precipitation by the copper method. The amino nitrogen determinations on Solution X before and after precipitation with phosphotungstic acid show that there is 1.81 per cent less amino nitrogen after the precipitation than before. This must be due to the exposed amino groups of the nitrogenous matter precipitated by the phosphotungstic acid, and although the difference involved the measurement of only 0.03 cc. of nitrogen gas in the micro determination, it was confirmed by repeated determinations which agreed practically as closely as the burette could be read. After hydrolysis there was about eight times as much amino nitrogen, which clearly indicates that the precipitate consisted chiefly of peptides of a less complex nature than protein. The amino nitrogen in the filtrate from the treatment of Solution X with phosphotungstic acid which amounted to 4.81 per cent probably originated from the *free* amino-acids in the extract. There is a much larger discrepancy between the percentage of total nitrogen precipitated by phosphotungstic acid and the sum of the percentages of ammonia and amino nitrogen after hydrolysis than may reasonably be accounted for by the non-amino nitrogen of the peptide compounds. This seems to indicate that there are other basic nitrogen compounds in flour extracts, whose nature is as yet largely unknown. A murexide test for purines resulted negatively.

An attempt was made to ascertain whether or not the copper method removes along with the proteins any appreciable amounts of amino-acids or peptide-like compounds of a nature less complex than that of the proteins by redissolving some of the copper-protein precipitate in glacial acetic acid and testing the solution for free amino groups since the presence of considerable copper does not interfere with the reaction between the amino groups of amino-acids and nitrous acid. Accordingly a solution of the copper precipitate containing 4 mg. of nitrogen was introduced into the Van Slyke micro-apparatus, but there was no evidence to show that any such compounds were present in the precipitate. Therefore, it is not believed that any serious error

from this source is introduced in the case of flour extracts, notwithstanding the fact that under certain conditions copper is known to be capable of forming insoluble compounds with a few amino-acids, though not with the majority of them.

When water extracts of flour were allowed to stand for several weeks at room temperature in the presence of toluene, and analyzed at intervals for non-protein nitrogen by the copper method, a gradual increase in non-protein nitrogen by autolysis occurred although there was no sign of putrefaction. The amount of total non-protein nitrogen doubled during the period from May 25 to July 6, 1917. This indicates that the method is applicable to proteolysis studies in flour.[6]

SUMMARY.

1. Practically a complete separation of protein from non-protein nitrogenous substances in water extracts of wheat flour may be accomplished by treating the extract with 0.1 N NaOH followed by 0.1 N $CuSO_4$ until there is but slightly more $CuSO_4$ than an exactly equivalent amount of NaOH. The method is simple of manipulation and leaves no troublesome excess of the reagents employed for the precipitation. The method permits of rapid filtration through ordinary filter paper, giving a water-clear solution which may be readily concentrated to one-twentieth its original volume, for determinations of amino nitrogen by Van Slyke's micro method, and for amide nitrogen determinations. Some peptide nitrogen is not precipitated by the copper method, but the removal of the true proteins is practically complete.

2. Normal patent flour contains but about 2 mg. of amino-acid nitrogen for every 100 gm. of flour, and about three times as much nitrogen in free acid amide form.

3. There is probably a considerable amount of *non-protein nitrogen* not precipitated by the copper method which is neither amino-acid nitrogen nor is it in the form of peptide complexes; its nature is not known.

4. The method is applicable to studies of proteolysis or other studies involving the estimation of protein cleavage products in wheat flour.

5. It is not unlikely that the method will be found applicable to biological extracts from other sources than wheat and flour.

[6] Acknowledgment is made to Miss Erma Lessel, who performed a large part of the preliminary analytical work.

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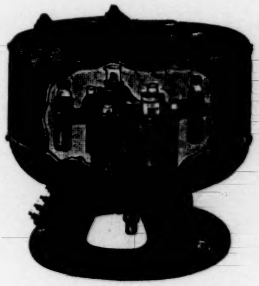

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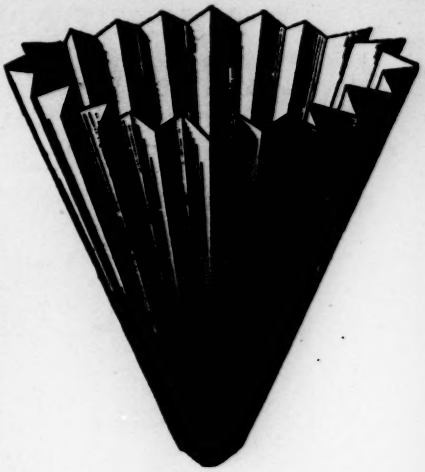

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